EVALUATION OF LEAF EXTRACTS OF *Simarouba glauca* ON EXPERIMENTALLY INDUCED INFLAMMATORY BOWEL DISEASES IN WISTAR RATS

Dissertation Submitted to

The TamilNadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the award of the Degree of

MASTER OF PHARMACY

(Pharmacology)

OCTOBER-2016

DEPARTMENT OF PHARMACOLOGY

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPPATTI ROAD,

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Under the Guidance of
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CERTIFICATE

This is to certify that the dissertation work entitled "**Evaluation of leaf extracts of Simarouba glauca on Experimentally induced Inflammatory Bowel Diseases in wistar rats"** is a bonafide research work carried out by the candidate (**RegNo: 261425819)** and submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2015-2016.

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Date: **Guide**

Department of pharmacology

DECLARATION

I do hereby declare that the dissertation work entitled **"Evaluation of leaf extracts of *Simarouba glauca* on Experimentally induced Inflammatory Bowel Diseases in wistar rats"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy in Pharmacology**, was done by me at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2015-2016.

Date: **Reg No: 261425819**

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Date:

Internal Examiner **External Examiner**

Convener of Examinations

Examination Centre: KMCH College of Pharmacy, Coimbatore

ABSTRACT

Aim: The aim of the study was to evaluate the Inflammatory Bowel Disorder activity of EASG&CHSG extracts of *Simarouba glauca* on Experimentally induced Inflammatory Bowel Disease in wistar rats. ***Methods:*** The levels of phytochemicals were quantified and HPTLC study was conducted using β-sitosterol as reference. *In vitro* antioxidant property of EASG & CHSG was evaluated using DPPH and ABTS assay. *in vitro* anti activity of EASG & CHSG was investigated against MTT assay on caco2 cell lines. Evaluation of IBD was done in wistar rats. Rats were treated with indomethacin (7.5mg/kg,s.c ,6% glacial acetic acid(0.1ml,intrarectal) for 3days after 14 days pretreatment with extract and standard drug (prednisolone 10mg/kg). The MPO Level, macroscopic features scoring, body weight analysis and microscopic (histopathological study was carried out and evaluated. Antioxidant potential of intestinal tissue was determined by measuring activities of Superoxide Dismutase, Catalase, Glutathione peroxidase, Lipid per oxidation and reduced Glutathione. ***Results:*** EASG & CHSG possessed considerable amount of phytoconstituents such as flavonoid, phenols and β-sitosterol; and also significantly scavenged the free radicals making it a potent antioxidant. The results of macroscopic scoring and MPO level activity suggest that EASG & CHSG at 400mg/kg significantly ($p < 0.001$) reduced the inflammation in IBD induced animals in both the models. In *In vitro* cytotoxicity study , a significant reduction in cell damage was observed when caco2 cells were pretreated with EASG and CHSG extracts when compared to control. EASG & CHSG at 400mg/kg significantly reduced lipid per oxidation and increased the Superoxide Dismutase(SOD), Catalase(CAT) and Glutathione peroxidase(GPx).Reduced Glutathione(GSH) levels and produced significant antioxidantactivity**.** ***Conclusion:*** Attributing to the potent antioxidant and anti-inflammatory activity, we could conclude that the EASG & CHSG of *Simarouba glauca* showed significant anti- inflammatory activity due to presence of β-sitosterol and antioxidants such as phenols and flavonoids.

Keywords: Ethylacetate extract of *Simarouba glauca* (EASG) & Chloroform extract of *Simarouba glauca* (CHSG), Inflammatory Bowel Disease (IBD), Myeloperoxidase(MPO)

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3.1. MATERIALS USED FOR THE STUDY

Table 1: List of instruments

Sl.No	Instruments	Manufacture
1	Analytical weighing balance	Shimadzu
2	Cooling centrifuge	Remi
3	Deep freezer (-80^0 C)	Remi
12	Digital Vernier Caliper	Absolute digimatic
11	Electric water bath	Technico
4	Homogenizer	Remi
5	Hot air oven	Narang Scientific works
6	HPTLC	Camag
7	pH meter	Eutech
10	Rotary evaporator	IKA RV10
8	Ultra sonicator	Soltec
9	UV Spectrophotometer	PharmaspecUV-1700,Shimadzu

Table 2: List of chemicals

SL.No	Chemical	Manufacturer
1	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)Diammonium salt(ABTS)	Himedia
2	5,5-dithiobis (2-nitro benzoic acid) DTNB	Himedia
3	Ascorbic acid	SISCO
4	Bovine serum albumin(BSA)	SD- Fine chem.
5	1,1-diphenyl-2-picrylhydrazyl(DPPH)	Sigma Aldrich
6	Folin-Ciocalteu reagent	SD-Fine chem.
7	Gallic acid	Sigma Aldrich
8	Glutathione reduced	Himedia
9	Sodium azide	Himedia
10	Thiobarbituric acid(TBA)	Himedia
11	Trichloric acetic acid(TCA)	Himedia
12	Quercetin	Himedia
13	Indomethacin	Microlabs
14	6% glacial acetic acid	Himedia

3.2.METHODS

3.2.1. PLANT COLLECTION AND AUTHENTICATION

Simarouba glauca was collected from Alapuzha district,Kerela and authenticated from Botanical survey of India (BSI) Southern circle,Coimbatore Tamil nadu.The authentication certificate number is No.BSI/SRC/5/23/2016/tech/604 .Soon after collection ,the leaves were dried in the shade and crushed to coarse powder for extraction**.(41)(42).**

3.2.2. EXTRACTION OF THE PLANT MATERIAL

The leaves were first treated with petroleum ether , the defatted material was then extracted with ethyl acetate and chloroform using soxhlet apparatus. After extraction the solvent was evaporated by using rotary evaporator and dried at room temperature to give viscous mass. The crude extracts were weighed and stored at 4^0C before analysis.

3.2.3. QUALITATIVE PHYTOCHEMICAL ANALYSIS OF CHSG AND EASG.

Preparation of test sample

A small quantity of extract was dissolved in 5ml of distilled water and then filtered.The filtrate was tested to detect the presence of different phytochemical constituents in the sample.

3.2.3.1. TEST FOR CARBOHYDRATES

- **Molisch's test:**
  To 2-3ml of filtrate,few drops of Molisch's reagent was added.Then concentrated sulphuric acid was added along the sides of test tube.Appearance of violet colour ring at the junction of two liquids infers the presence of carbohydrates.
- **Fehling's test**
  1ml of Fehling's-A(copper sulphate in distilled water) was mixed with 1ml Fehling's-B(Potassium tartarate and sodium hydroxide in distilled water) Solutions in a test tube and was boiled for one minute. 1ml of filtrate was added and heated gently.Formation of brick red precipitate indicates the presence of reducing sugars.

- **Benedict's test**

Equal volume of filtrate and Benedict's reagent (alkaline solution containing cupric citrate complex) was mixed and heated in boiling water bath for 5min.Formation of reddish brown precipitate infers the presence of reducing sugars.

3.2.3.2. TEST FOR ALKALOIDS

Small amount of extract was mixed with few ml of dilute.hydrochloric acid.shaked well and filtered.The filtrate was used to perform the following tests:

- **Dragendroff's test**

To 2-3ml of filtrate, few drops of dragendroff's reagent(potassium bismuth iodide solution) was added. Formation of orange red precipitate indicates the presence of alkaloids

- **Mayer's test**

To 2-3ml of filtrate, few drops of Mayer's reagent (potassium mercuric iodide solution) was added.Cream (dull white) precipitate are formed.

- **Wagner's test**

To 2-3ml of filtrate, few drops of Wagner's reagent (solution of iodine in potassium iodide) was added. Reddish brown precipitate was obtained.

- **Hager's test**

To 2-3mlof filtrate, few drops of Hager's reagent was added. Yellow precipitate was obtained.

3.2.3.3. TEST FOR TRITERPENOID

- **Libermann-Burchard test:**

To the extract few drops of acetic anhydride was added, followed by few drops of concentrated sulphuric acid. A brown ring formed at the junction of two layers and the upper layer turned green colour, infers the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoid.

- **Salkowski test:**

To small quantity of the extract chloroform and few drops of concentrated sulphuric acid was added and allowed to stand for few minutes. Yellow colour at the lower layer indicates the presence of triterpenoids.

3.2.3.4. TEST FOR GLYCOSIDES

- **Legal's test**

To the extract, 1ml of pyridine and 1ml of sodium nitroprusside was added. Pink to red colour appeared.

- **Keller-Killiani test**

To 2ml extract, glacial acetic acid, trace quantity of ferric chloride was added and 2 to 3 drops of concentrated sulphuric acid was added. Reddish brown colour appeared at the junction of two liquid indicates the presence of cardiac glycosides.

- **Baljet test:**

To 2ml of extract sodium picrate solution was added. Yellow to orange colour formation indicates the presence of glycosides.

3.2.3.5. TEST FOR STEROIDS AND STEROLS

- **Liebermann- Burchard reaction**

2ml of extract was mixed with chloroform. 1-2ml of acetic anhydride and 2 drops of concentrated sulphuric acid was added along the sides of the test tube. The solution became red, then blue and finally bluish green colour.

- **Salkowski reaction**

To 2ml of extract, 2ml chloroform and 2ml concentrated sulphuric acid was added. Shaken well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

3.2.3.6. TEST FOR PHENOLS

- **Ferric chloride test**

To 1ml of the alcoholic solution of the extract, 2ml of distilled water followed
by few drops of 10% ferric chloride was added. Formation of blue or green colour indicates the presence of phenols.

- **Lead acetate test**

1ml of alcoholic solution of extract was diluted with 5ml distilled water and to this
few drops of 1% aqueous solution of lead acetate was added. A yellow colour precipitate was formed.

3.2.3.7. TEST FOR TANNINS

- **Lead acetate test**

To 5ml of aqueous extract, few drops of lead acetate was added. A yellow or red colour precipitate was formed.

3.2.3.8. TEST FOR SAPONINS

- **Foam Test:**

1 ml of test sample was diluted with 20 ml of distilled water and shaked it in a graduated cylinder for 3 minutes. Foam of 1 cm after 10 min indicates the presence of saponins.

- **Froth test:**

To 5 ml of test sample few drops of sodium bicarbonate was added. The mixture Was shaked vigorously and kept it for 3 minutes. A honey comb like froth formation indicates the presence of saponins.

3.2.3.8. TEST FOR FLAVONOIDS

- **Zinc hydrochloride reduction test**

The extract with mixture of zinc dust and concentrated hydrochloric acid was treated.Formation of red colour indicates the presence of flavonoids.

- **Alkaline reagent test**

To the extract, few drops of sodium hydroxide solution was added. Formation of an intense yellow colour, which turns to colourless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

3.2.3.9. TEST FOR PROTEINS AND AMINO ACIDS

- **Biuret test**

To 3ml of test solution 4% sodium hydroxide was added and few drops of 1% copper sulphate solution. Formation of violet colour indicates the presence of proteins.

- **Ninhydrin test**

3ml of test solution and 3drops of 5% Ninhydrin solution was heated in boiling water bath for 10min. Formation of purple or bluish colour indicates the presence of free amino acids**.(43)(44).**

3.2.4. HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHIC (HPTLC) METHOD FOR ESTIMATION OF STEROIDS.

High performance thin layer chromatography (HPTLC) is a suitable quality assessment tool for the evaluation of herbal medicines and natural drug. Additionally, numerous samples can be run in a single analysis thereby it will reduce the analytical time. With HPTLC, the same analysis can be view that single and different wavelength of light; thereby providing a more complete profile of the plant and it is typically observed with more specific types of analysis.

Experimental conditions

- Stationary phase : Aluminium plates precoated with Silica Gel 60F254 (10cm×10cm×0.2 mm thickness)
- Mobile phase :Acetone:hexane(3:9)
- Sample for HPTLC : CHSG,EASG and standard steroid solution
- Sample application : Camag Linomat 5
- Chamber type : Twin trough Chamber 10× 10 cm
- Chamber saturation : 5 min
- Development time : 30 min
- Development distance : 7 cm
- Detection : Camag Scanner 3
- Data system : win CATS Planar Chromatography Manager.

Instrumental Parameters

- Number of track : 9
- Band length : 6.0 mm
- Application position : 10 mm
- Solvent front position : 80.0 mm
- Solvent volume :10 ml
- Position of first track : 10 mm
- Distance between tracks : 10.0 mm
- Scan start position Y : 5.0 mm
- Scan end position : 75.0 mm
- Slit dimension : 6.00 × 0.45 mm, Micro

- Optimized optical system : light
- Scanning speed : 20 mm/sec
- Data resolution : 100 µm/ step

Measurement table:

- Wavelength : 366 nm
- Lamp : D2 & W
- Measurement type : Remission
- Measurement mode : Absorption
- Optical filter : Second order
- Detector mode : Automatic

Preparation of standard

A stock solution of β-sitosterol (1mg/ml) was prepared in methanol. Different volumes of stock solution 1µl,3µl,5µl,7µl,10µ,12µl,15µl were spotted on TLC plate to obtain concentration of 0.001,0.003,0.005,0.007,0.01,0.012,0.015 mg/spot of β-sitosterol.

Preparation of test sample

Weighed accurately 1g of both CHSG and EASG and dissolved separately in 10 ml of chloroform and ethylacetate respectively. Each sample was filtered using WhatmannNo.1 filter paper.10µl of both extracts were spotted on TLC plate.

Principle: The fundamental mechanisms are the partitioning of the moving compounds between two liquid phases and also their being reversely bound on the surface of the adsorbent. Adsorption denotes the enrichment of a gaseous or dissolved material at a phase boundary (Surface of a solid). The effects of adsorption are particularly marked when the interface was large. The separation achieved in absorption chromatography is strongly dependent upon the surface of the powdered adsorbent.

When, a mixture of compounds is spotted on a HPTLC plate, the compound which was soluble and not strongly adsorbed moves up fast along with the solvent. Those which are not so soluble are more strongly absorbed moves up less, readily leading to the separation of the compound. The behavior of the compound is dependent upon both adsorbent and solvent**.(45)**

Procedure:

The samples; β-sitosterol 1µl,3µl,5µl,7µl,10µ,12µl,15µl and CHSG and EASG 10µl and 10µl were spotted in form of bands with a Camag microlite syringe on pre-coated Silica Gel glass plate 60F254 (10×10 cm with 0.2 mm thickness) using a Camag Linomat 5 applicator. The plates were pre-washed with methanol and activated at 60^{o}C for 10 min prior to chromatography. The sample loaded plate was kept in TLC twin trough developing chamber after chamber saturation with respective mobile phase. The optimized chamber saturation time for mobile phase was 5 min at room temperature. Linear ascending development was carried out and the plate was developed in the respective mobile phase up to 7 cm. The developed plate was then dried by hot air oven for the development of bands . The dried plate was observed under UV light at 254nm and 366nm and photo documentation was done. Densitometric scanning was performed on Camag TLC scanner 3 in the absorbance mode at 366nm. The percentage of active ingredient(β-sitosterol) present in the SG leaf extract was compared with the standard**.(45)(46)(47)**

3.2.5. QUANTIFICATION OF TOTAL PHENOLS AND FLAVONOIDS

3.2.5.1. ESTIMATION OF TOTAL PHENOLS

Reagents

- Folin-Ciocalteu's reagent
- 20% sodium carbonate

Procedure

The total phenols of the EASG and CHSG were determined by the Folin-Ciocalteau assay method. An aliquot (1000µl) of extract (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100µg/ml) were mixed with 50µl of Folin-ciocalteau reagent and 860µl of distilled water and incubated for 5min at room temperature. Then100µl of 20% sodium carbonate and 890µl of distilled water were added to the above mixture and kept in dark for 30 min to complete the reaction. After incubation, absorbance of the mixture was measured against blank at 725nm. Distilled water was used as reagent blank. The calibration curve of Gallic acid was prepared. All the tests were performed in triplicate in order to get mean the values. The total phenol content was expressed as milligrams of Gallic acid equivalents (GAE) per gram of extract**.(48)**

3.2.5.2. ESTIMATION OF TOTAL FLAVONOIDS

Reagents

- Methanol
- 10% aluminium chloride
- 1M Potassium acetate

Procedure

The total flavonoid content of the EASG and CHSG was determined by using Aluminium chloride colorimetric method. An aliquot (1ml) of extract (1mg/ml) or standardsolutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml)in methanol were separately mixed with 1ml of methanol,0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. Shaken well and incubated at room temperature for 30 min. After incubation, absorbance of the reaction mixture wasmeasured at 415nm. Methanol (2ml) was used as reagent blank. To substitute 10%aluminium chloride, 100µl of distilled water was added to the blank. The calibration curve of Quercetin was prepared. All the tests were performed in triplicate in order to get the mean values. The total flavonoid content was expressed as milligrams of Quercetin equivalents per gram of extract**.(48)**

3.2.6. INVITRO-ANTIOXIDANT STUDIES OF EASG & CHSG.

3.2.6.1. DPPH FREE RADICAL SCAVENGING ASSAY

Principle

The molecule of 1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol/methanol solution centred at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

Z• + AH = ZH+ A•.(**49)**

Procedure

The antioxidant activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical (Blois method).0.3mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 1ml of various concentrations of sample and the reference compound (10, 20, 30, 40 and 50 µg/ml), were shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm against a blank. Reference compound used here was ascorbic acid. A control reaction was carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Antiradical activity was expressed as percentage inhibition (I %) and calculated using the following equation**.(48).**

Percentage inhibition (I %) = (Abs control- Abs sample /Abs control) X 100

Different sample concentrations were used in order to obtain calibration curves and to calculate the EC50 values. (EC50 - concentration required to obtain a 50% radical scavenging activity).

3.2.6.2.**ABTS RADICAL CATION ASSAY**

Principle

ABTS decolourization assay is an inhibition method. The peroxidase substrate 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), forms a relatively stable radical (ABTS+) upon one electron oxidation. This assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical and inhibits the absorption of the radical cation which has characteristic long-wavelength absorption spectrum showing maxima at 660,734, and 820nm.The relatively stable ABTS radical has a green colour and is quantified spectrometrically at 734nm**.(50)**

Procedure

ABTS radical scavenging activity of the extract was measured by Rice-Evans method. ABTS was dissolved in water to a 7mM concentration. ABTS radical cation (ABTS+) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734nm and equilibrated at 300c. After addition of 2ml of diluted ABTS+ solution to 20µl of various concentrations of sample or reference compound (ascorbic acid), the reaction mixture was incubated for 6min and then absorbance was measured at 734 nm against a blank. A control reaction was carried out without the sample. All the tests were performed in triplicate in order to get the mean values. The percentage inhibition of $ABTS^{+}$ by the sample was calculated according to the formula:

Percentage inhibition (I %) = (Abs control- Abs sample /Abs control) X 100

Different sample concentrations were used in order to obtain calibration curves and to calculate the EC50 values. (EC50 - concentration required to obtain a 50% radical scavenging activity).**(48)**

3.2.7. *INVITRO* CYTOTOXICITY STUDY OF EASG & CHSG.

Reagents used:

- 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT),
- Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS),
- Modified Eagle's Medium (MEM) and Trypsin(from Sigma Aldrich Co, St Louis, USA).
- EDTA.
- Glucose and antibiotics (from Hi-Media Laboratories Ltd., Mumbai)
- Dimethyl Sulfoxide (DMSO) and Propanol (from E.Merck Ltd., Mumbai, India).

Cell lines and Culture medium

CaCO2 cell line was selected as an invitro cell line**(51)(52)** and was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in MEM supplemented

with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with Trypsin solution (0.2% trypsin, 0.02% EDTA in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For Cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with MEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

Principle: The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using MEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a

wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line**.(53)**

$$\% \text{ Growth Inhibition} = 100 - \left(\text{Mean OD of individual test group} \quad X\ 100 \right)$$

3.2.8.ACUTE TOXICITY STUDY.(35)

Based on previously conducted study of *Simarouba glauca*,the dose was selected.

3.2.8.1. PHARMACOLOGICAL STUDY

Experimental animal

Male wistar rats of 6-8 weeks old and 160-180 g body weight were obtained from the animal house ,KMCH College of pharmacy, Coimbatore. All rats were kept at room temperature and allowed to accommodate in standard conditions at 12-hr light and 12-hr dark cycle in the animal house. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. The experimental procedure was approved by IAEC (Institutional animal ethical committee) of KMCH College of Pharmacy, Coimbatore-48 .Governed by CPCSEA Government of India. Proposal number: KMCRET/ M.PHARM/03/2015-16).

3.2.8.2.EVALUATION OF INFLAMMATORY BOWEL DISEASES ACTIVITY OF CHSG AND EASG.

PREPARATION OF DRUG SOLUTIONS

Standard drug

Prednisolone was used as a standard drug. The drug was dissolved in normal saline and administered orally using gavage needle at a dose of 10mg/kg daily for 3days.

Preparation of extracts

CHSG and EASG was weighed and dissolved in chloroform and Ethylacetate respectively, and it was administered orally to the rats at a dose of 400mg/kg each of CHSG and EASG daily upto 14days.The normal control group received only normal saline .

3.2.8.2.a. INDOMETHACIN- INDUCED ENTEROCOLITIS.

Principle: Indomethacin, a non-selective COX inhibitor produces enterocolitis .Prolonged use of Indomethacin is known to induce enterocolitis with pathological features such as transmutable ulcers, wall thickening, adhesions, granulomatous inflammation, crypt abscesses and fibrosis in jejunum-ileum region. The features are similar to those found in Crohn's disease . Multiple factors namely hepatobiliary export of glucuronides of Indomethacin down regulation of heat shock proteins , endoplasmic reticulum stress , and deficiency of endogenous prostaglandins have been identified to be involved in pathogenesis of Indomethacin induced Entrocolites . Increased Oxidative stress, mitochondrial dysfunction and inflammation are found to be prominent factors that cause destructive changes**.(55)**

Table3:EXPERIMENTAL DESIGN FOR INDOMETHACIN-INDUCED ENTEROCOLITIS

Animals were divided into 5 groups, each group consists of 6 rats**.(54)(31)**

Group	Treatment
Group I: normal	Rats received only normal saline(1ml,p.o) for 14 consecutive days
Group II: control	Rats received indomethacin(7.5mg/kg,s.c), on two consecutive days;
Group III: Standard	Rats received prednisolone (10mg/kg,p.o), for 3days and indomethacin (7.5mg/kg,p.o) for 2days.
Group IV: CHSG and EASG(400mg/kg)	Rats received 14days pretreatment with CHSG and EASG and indomethacin (7.5mg/kg,s.c), for 3 days
Group V: CHSG and EASG (400mg/kg)	

Procedure:

Rats (200-250g) were given indomethacin 7.5mg/kg subsutaneously on two consecutive days. On the third day after 24 hours fasting the animals were sacrificed by cervical dislocation and dissected open to remove GIT (from stomach to anus). GIT s was flushed gently and cut open.

Duedenum,jejunum,ileum,caecum and colon were scored for inflammation based on their macroscopic features.Tissues were fixed in 10% formalin saline and examined histopathologically.

Quantification of inflammation was done using myeloperoxidase assay**.(31)(55)(56)**

3.2.8.2.b. ACETIC ACID -INDUCED ENTEROCOLITIS IN RATS

Principle: Intrarectal administration of dilute solution of acetic acid causes non-transmural inflammation characterized by increased neutrophil infiltration into the intestinal tissue, massive necrosis of mucosal and submucosal layers, vascular dilation, edema and submucosal ulceration that are noteworthy features of human colitis . Acetic acid cause damage to distal colon portion, The mechanisms by which acetic acid produces inflammation appear to involve the entry of the protonated form of the acid into epithelium, where it dissociates to liberate protons within intracellular acidification that are likely accounts for the epithelial injury**.(13)**

Table 4: EXPERIMENTAL DESIGN FOR ACETIC ACID INDUCED ENTEROCOLITIS

Animals were divided into 5 groups, each group consists of 6 rats**.(56)**

Group	Treatment
Group I: normal	Rats received only normal saline(1ml,p.o) for 14 consecutive days
Group II: control	Rats received 0.1ml of 6% acetic acid solution intrarectally.
Group III: Standard	Rats received prednisolone (10mg/kg,p.o), for 3days and 0.1ml of 6% acetic acid solution for 2days.
Group IV: CHSG and EASG(400mg/kg)	Rats received 12days pretreatment with CHSG and EASG and 0.1ml of 6% acetic acid solution,intrarectally on 13^{th} and 14^{th} day.
Group V: CHSG and EASG (400mg/kg)	

Procedure:

Rats (200-250g) were used for the study. overnight fasted animals both the treated and control group animals were instilled with 0.1ml of 6% acetic acid solution into the rectum of the rats .Animals were allowed to hang in air by holding their tails for 1-2min.This prevented the spillage of the solution from the rectum

After 48hours animals were Sacrificed by cervical dislocation and dissected to remove the colon and caecum. Colon and Caecum was flushed gently with saline,cut open and scored for inflammation based on the macroscopic features.Tissues were fixed in 10% formalin saline and examined histopathologically.

Quantification of inflammation was done using myeloperoxidase assay**.(31)(57)(56).**

EVALUATION OF PARAMETERS OF INFLAMMATORY BOWEL DISEASE.

The disease induced induced experimental animals was evaluated based on its macroscopic and microscopic characteristics. Evaluation pattern for macroscopic characteristics was used after some modifications.The inflammation was quantified using myeloperoxidase assay.

1. **Assessment of Myeloperoxidase activity:** myeloperoxidase enzyme activity was determine to measure the inflammation in the intestinal tissues.It was measured by using DTNB method**.(58)**
2. **Assessment of oxidative stress:** Oxidative stress was determined using Lipid peroxidation levels in the Intestinal mucosa as an indicator of stress level. The intestinal mucosa was scraped out from each animal and homogenized with potassium phosphate buffer**. (55).**

3.Histological evaluation:-

A portion of the intestine of each rat was collected and fixed in 10 % Buffered neutral formalin. Sections were stained with haematoxylin and eosin**(31)**

3. **Scoring of the animal intestinal tissues**; The intestinal tissues, 5 cm proximal duodenum, 10 cm distal jejunum, 10 cm proximal ileum, whole caecum and 5 cm proximal colon were isolated and were scored visually for inflammation based on macroscopic features using following pattern**.(31)(56)(54)**

Table 5: Scoring pattern for rat duodenum, jejunum, ileum, caecum and colon in indomethacin induced enterocolitis(31)(56)(54)

Score	Macroscopic changes
0	No visible change
1	Hyperemia at sites
2	Loss of mucosal integrity
3	Lesions having diameter 1 mm or less
4	Lesions having diameter 2 mm or less (no<5)
5	Lesions having diameter 2 mm or less (no 5-10)
6	Lesions having diameter 2 mm or less (no>10)
7	Lesions having diameter more than 2 mm (no<5)
8	Lesions having diameter More than 2 mm (no 5-10)
9	Lesions having diameter more than 2 mm (no>10)

Scoring for rat caecum and colon:Rat caecum and colon(5cm long) was scored for macroscopic feature using following scoring pattern**.(31)(56)**

Table 6: Scoring pattern for rat caecum and colon in acetic acid induced enterocolitis

Score	Percentage area affected
0	0
1	1-5
2	5-10
3	10-25
4	25-50
5	50-75
6	75-100

Samples were isolated and washed with normal saline and stored for in vivo antioxidant studies. The tissues were homogenized with motor driven Teflon coated homogenizer in ice-cold (10% w/v) 0.1 M Tris-HCl buffer pH 7.4 to get 10% homogenate. The homogenate was centrifuged at

10000 rpm for 10 min at 5˚C. The supernatant was collected and used for following in vivo studies**.(31)**

3.2.8.3.MYELOPEROXIDASE ASSAY FOR QUANTIFICATION OF INFLAMMATION*:*

Principle: Mpo activity enzyme is a measure of leukocyte infiltration.It oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as an oxidizing agent. The enzyme involves in the nitration of tyrosine residues,which cause variety of inflammatory disorders**.(59)**

Procedure:

Pieces of inflamed tissues were taken.The tissues was then rinsed with saline,blotted dry, weighed and excised.Minced tissue was homogenized in phosphate buffer (pH 7.4), using remi tissue homogenizer.The homogenate was centrifuged at 10000 rpm for 20min at 4^0C. supernatant was discarded,myeloperoxidase activity was measured by DTNB method as follows:

0.1ml of tissue homogenate was treated with 0.1ml of TNB and 1ml of sodium acetate buffer followed by addition of about 0.8ml of Nacl solution and 35µl of hydrogen peroxide.The change in absorbance was measured spectrophotometrically at 490nm**.(58)**

One unit of MPO activity is defined as the change in absorbance per minute by 1.0 at room temperature, in the final reaction**.(31)**

3.2.8.4. *IN VIVO* ANTIOXIDANT ACTIVITY

3.2.8.5. Preparation of tissue homogenate

For the estimation of non-enzymatic and enzymatic antioxidants, tissue was minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 min and the resulting supernatant was used for enzyme assays**.(31)**

3.2.8.4.1. ESTIMATION OF PROTEINS:

Reagents

· Alkaline copper reagent

· Solution A: 2% sodium carbonate in 0.1 N NaOH.

· Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate

· Solution C: 50 ml of solution A was mixed with 1 ml of solution B just before use.

· Folin's phenol reagent (commercial reagent, 1:2 dilutions) Bovine serum albumin (BSA).

Principle:

This method involves two steps;

Step: 1- -protein binds with copper in alkaline medium and reduces it into cu++.

Step: 2- the cu++ formed catalyses the oxidation reaction of aromatic amino acids by reducing phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue colour and absorbance was measured at 640 nm**.(60)**

Procedure:

0.1 ml homogenate was made up to 1 ml with distilled water and to this; 5 ml of alkaline solution was added, mixed well and allowed to stand for 10 min. Then a volume of 0.5 ml Folin's reagent was added, mixed well and incubated at room temperature for another 10 min. The blue colour developed was measured at 660 nm against blank. Bovine serum albumin (1 mg/ml) served as the standard and form the standard graph obtained; the amount of protein in the sample was calculated and expressed as mg/100 mg tissue.

3.2.8.4.2. ENZYMATIC ANTI-OXIDANT ACTIVIY

3.2.8.4.2a. ESTIMATION OF CATALASE (CAT)

Reagents

· Dichromate acetic acid reagent (5% potassium dichromate + glacial acetic acid were mixed at 1: 3 ratio (v/v).

· 0.01M Phosphate buffer (pH-7.0).

· 0.2M hydrogen peroxide.

Principle:

The normal antioxidant activity of the enzyme catalase is due to acceleration of decomposition of hydrogen peroxide to water and oxygen. This method is based on the principle that by measuring the rate of decomposition of hydrogen peroxide by the enzyme catalase spectrophotometrically at 570 nm, since hydrogen peroxide has the absorbance at this range**.(61)**

Procedure:

To 1 ml of tissue homogenate 4 ml of hydrogen peroxide and 5 ml of phosphate buffer was added and mixed well. From this, 1 ml of solution was taken and mixed with dichromate acetic acid reagent and allowed to incubate for 30 min at room temperature. The absorbance was measured at 570nm. The activity of catalase was expressed as μ mole of H_2O_2 consumed /min/mg protein.

3.2.8.4.2b. ESTIMATION OF SUPEROXIDE DISMUTASE (SOD)

Reagents

· 50mM tris Hcl buffer at pH 8.2

· 10mM pyrogallol

· 10mM Hcl

Principle:

Pyrogallol autoxidizes rapidly in aqueous solution, where the reaction will be faster at higher pH, and leads to the formation of several intermediate products. Thus the solution first becomes yellow-brown with a spectrum showing a shoulder between 400 and 425nm. Molecular oxygen, carrying two unpaired electrons with parallel spins, has a preference for univalent reduction because spin restrictions arise when reduction with electron pairs is attempted. The recently discovered enzyme superoxide dismutase rapidly dismutases univalently reduced oxygen $O_2.^-$ i.e., the superoxide anion radical ($2O_2.^-$, + 2 $H^+ \longrightarrow$ O_2+ H_2O_2). The enzyme has proven to be a useful probe for studying the participation of the radical in reactions involving oxygen such as autoxidations. Thus O_2^-. has been shown to be involved in the autoxidation of e.g. sulphite, adrenalin and 6-hydroxydopamine**.(62)**

Procedure

This method might be used for determination of antioxidant activity of a sample, and it was described by McCord and Fridovich. The main purpose of this method that was estimated 5% of tissue homoginate after adding 75mM, 30mM, and 2mM from Tris-HCL (pH 8.2), EDTA, and pyrogallol respectively. Then, the absorbance was measured at 420nm. The percentage of inhibition was calculated depending on that the ability of enzyme to inhibit of oxidation. So, any

changes might be happened on the absorbance, it will give a clear picture on the ability of enzyme activity to prevent oxidation**.(63)**

3.2.8.4.2c. ESTIMATION OF GLUTATHIONE PEROXIDASE (GPx)

Reagents

· 0.32 M Phosphate buffer, pH 7.0

· 0.8 mM EDTA

· 10 mM Sodium azide

· 3 mM reduced glutathione

· 2.5 mM H2O2

· 10 % TCA

· 0.3 M Disodium hydrogen phosphate

· DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

· Reduced glutathione

Principle:

The principle behind the assay is the reduction of hydrogen peroxide (H2O2), by glutathione peroxidase through simultaneous oxidizing of reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is again reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP+ which results in the decreased absorbance at 340 nm and recycling the GSH. The decrease in absorbance at 340 nm is directly proportional to the GPx concentration**.(64)**

Procedure

To 0.1 ml of the tissue homogenate, 0.2 ml of EDTA, sodium azide, hydrogen peroxide were added and mixed. Then 0.4ml of phosphate buffer was added and allowed to incubate at room temperature. The reaction was arrested by the addition of 0.5 ml of TCA. The reaction mixture was centrifuged at 5000 rpm for 10 min and the supernatant was collected. To 0.5ml of the supernatant 4 ml of disodium hydrogen phosphate and 0.5 ml of DTNB were added and the colour developed was read immediately at 420 nm. The activity of Glutathione peroxidase was expressed as μmoles of glutathione oxidized/min/mg.

3.2.8.4.3. NON ENZYMATIC ANTIOXIDANT ACTIVITY

3.2.8.4.3a. ESTIMATION OF REDUCED GLUTATHIONE (GSH)

Reagents

· 5% TCA

· 0.6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate

· 0.2 M Phosphate buffer, pH 8.0

Principle:

DTNB known as Ellman's reagent was developed for the detection of thiol compounds. DTNB and sulfhydryl groups present in glutathione (GSH) react to generate 2-nitro-5- thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow coloured product, GSH concentration in a sample solution can be determined by the measurement at 412 nm**.(65)**

Procedure:

To 1ml of the homogenate, 1ml of the TCA solution was added and centrifuged. The supernatant was collected and the precipitate formed was removed. To 0.5 ml of supernatant 2 ml of DTNB was added, the volume was made up to 3 ml with phosphate buffer. Then absorbance was read at 412 nm. The amount of glutathione was expressed as μg/mg protein.

3.2.8.4.3b. DETERMINATION OF LIPID PEROXIDATION

Reagents

- Thiobarbituric acid 0.37%
- 0.25 N HCL
- 15% TCA

Principle:

This assay is based on the reaction between Thiobarbituric acid with malonyldialdehyde which is formed as a result of polyunsaturated fatty acid oxidation. This reaction leads to the formation of pink coloured TBA-MDA complex which is measured at 532 nm**.(61)**

Procedure:

To 0.1 ml of the sample, 2 ml of TBA-TCA-HCL reagent (ratio of 1:1:1) was added mixed and kept in a water bath for 15 minutes. Afterwards the solution was cooled and supernatant was removed and the absorbance was measured at 535 nm against reference blank. The level of lipid peroxidase was given as n moles of MDA formed/mg protein.

3.2.9.HISTOPATHOLOGICAL STUDIES

The tissues duodenum,jejunum,ileum,colon and caecum of all rats were isolated and were fixed in 10% formalin solution. The tissues were trimmed and prepare 5μm thick paraffin sections and stained in haematoxyline and eosin.The stained sections were examined for any inflammatory changes like infiltration of cells, necrotic foci, damage to tissue structures like payers patches, damage to nucleus,etc**.(31)**

3.2.9.1.HISTOPHATOLOGICAL TECHINIQUES

Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

1) Collection of materials

Thin pieces of 3 to 5 mm, thickness were collected from tissues showing gross morbid changes along with normal tissue.

2) Fixation:

Kept the tissue in fixative for 24-48 hours at room temperature

The fixation was useful in the following ways:

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue, and
d) Prevents shrinkage
e) Common Fixatives: 10% Formalin

3) Haematoxylin and eosin method of staining:

Deparaffine the section by xylol 5 to 10 minutes and remove xylol by absolute alcohol. Then cleaned the section in tap water and stained with haematoxylin for 3-4 minutes and again cleaned under tap water. Allow the sections in tap water for few minutes and counter stained with 0.5% eosin until section appears light pink 15 to 30seconds), and then washed in tap water. Blotted and dehydrated in alcohol and cleared with xylol 15 to 30 seconds). Mounted on a Canada balsam or DPX Moutant and kept the slide dry and remove air bubbles**.(31)**

3.2.10. STATISTICAL ANALYSIS

The data's of all the parameters were analysed using the software SPSS version 16 software The data's of all parameters were analysed by means of one way ANOVA (Analysis of Variance) followed by Dunnet's test. The results were expressed as mean ± SEM.

4.RESULTS

4.1. EXTRACTIVE YIELD OF S*imarouba glauca* LEAF EXTRACTS

Percentage Yield of CHSG and EASG

Coarsely powdered *Simarouba glauca* aerial leaves were extracted with chloroform and Ethylacetate using soxhlet apparatus after deffating with petroleum ether and the percentage yield was found to be

Ethyl acetate extract 2.85%

Chloroform extract 5.12%

4.2. PRELIMINARY PHYTOCHEMICAL ANALYSIS

Table 7: Phytochemical Analysis of CHSG and EASG

Sl.No	Phytochemical constituents	CHSG and EASG
1	Carbohydrate	Positive
2	Alkaloids	Positive
3	Triterpenoid	Positive
4	Glycosides	Positive
5	Steroid and sterols	Positive
6	Phenols	Positive
7	Tannins	Positive
8	Saponins	Positive
9	Phenols	Positive
10	Proteins and Amino acids	Positive

4.3. HPTLC SCREENING ANALYSIS OF EASG & CHSG

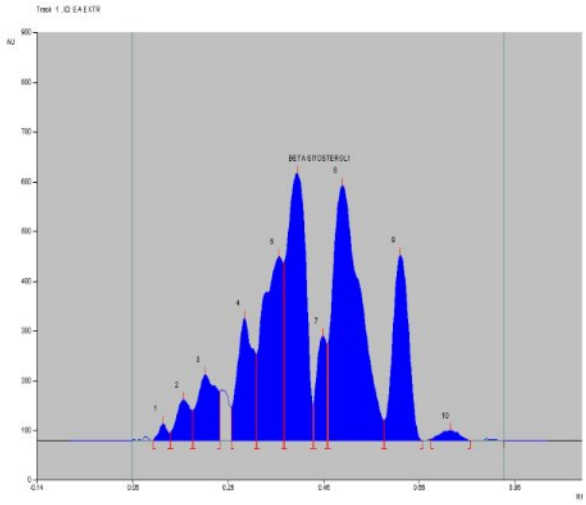
HPTLC study was carried out for the quantification of β-sitosterol in extract. Visualization was performed as done (figure 5) and the selected mobile phase gave Rf values(0.40) for β-sitosterol . After development the plate was scanned in densitometer under 366nm and the chromatogram obtained is depicted in figure 6. The calibration curve was prepared by plotting the concentration of β-sitosterol versus average area of peak (table 9, figure 8). The amount of β-sitosterol present in the extract was computed from calibration curve (Table 10).

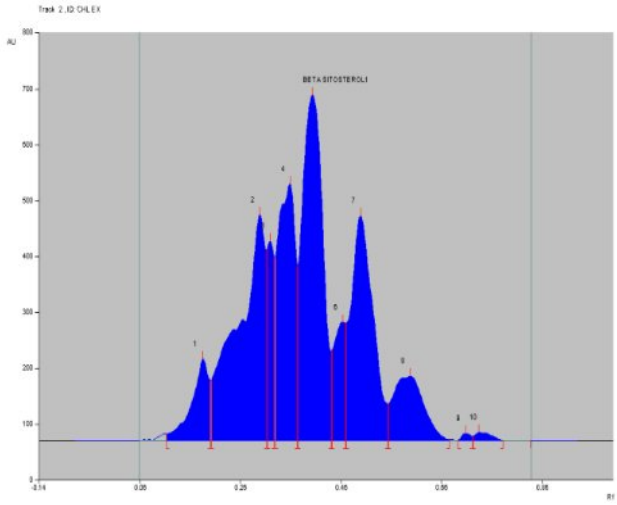
Figure 5: Detection of bands

Table 8:Lists of spots applied on HPTLC plate

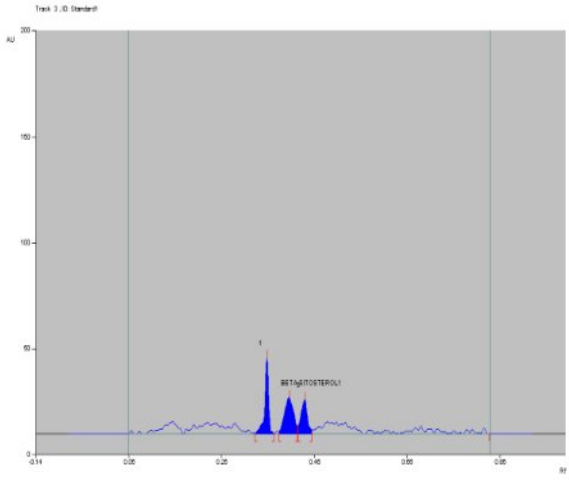
Track number	Sample
T1	EASG(10µl)
T2	CHSG(10µl)
S1	β-sitosterol(1µl)
S2	β-sitosterol(3µl)
S3	β-sitosterol (5µl)
S4	β-sitosterol(7µl)
S5	β-sitosterol(10µl)
S6	β-sitosterol(12µl)
S7	β-sitosterol (15µl)

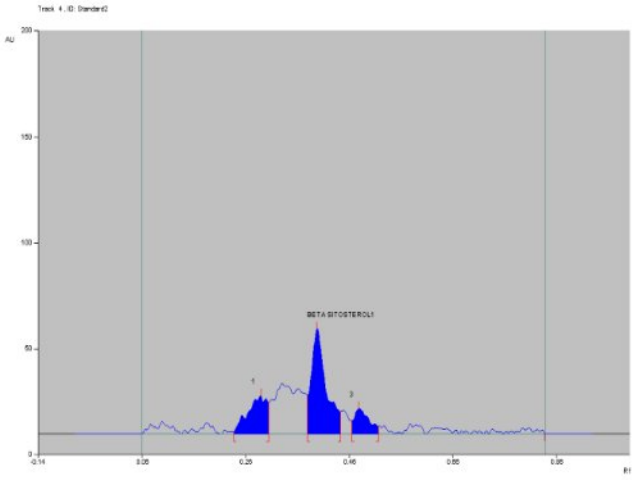
Figure 6: Chromatogram of β-sitosterol and CHSG & EASG



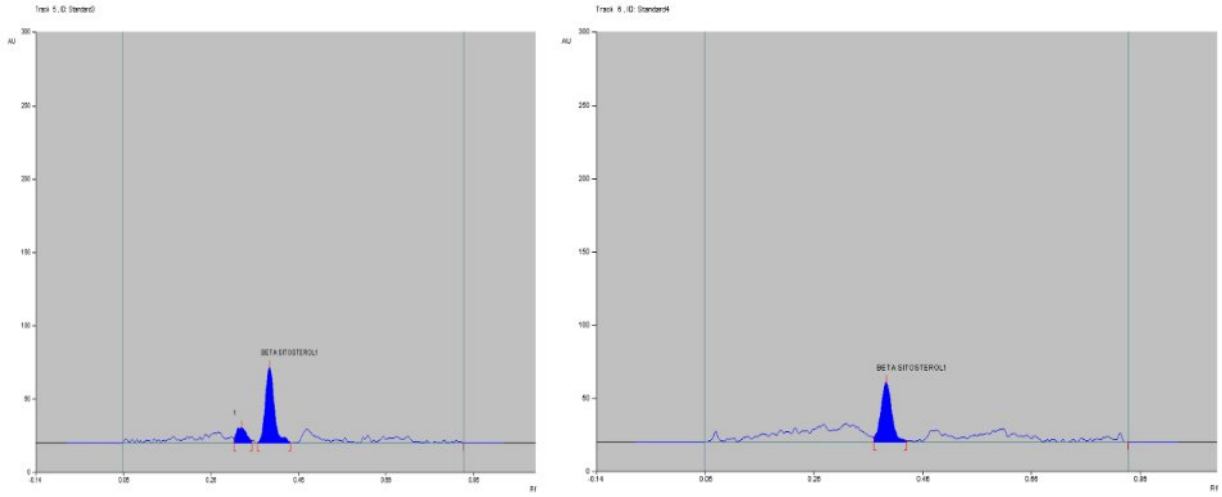


1: chromatogram of EASG (10µl) **2: chromatogram of CHSG (10µl)**

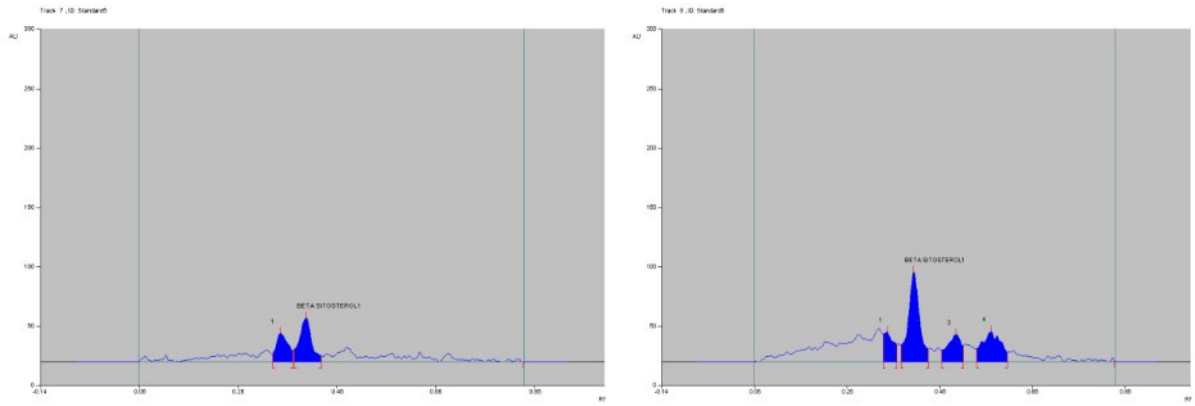




3: chromatogram of β-sitosterol(1µl) **4: chromatogram of β-sitosterol(3µl)**

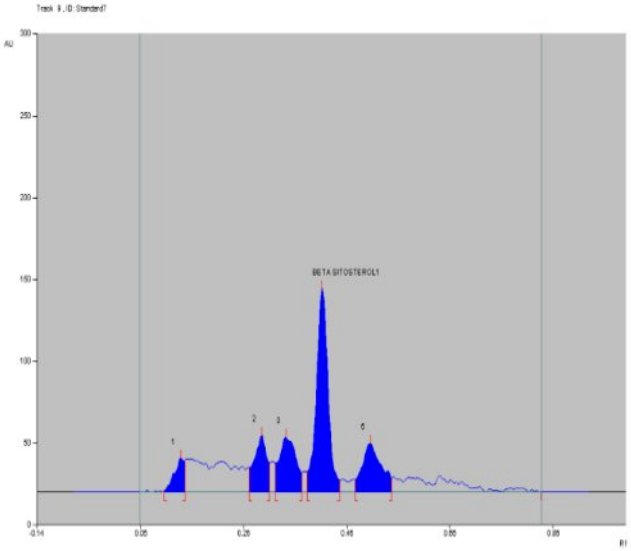


5:Chromatogram of β-sitosterol(5µl) 6:chromatogram of β-sitosterol(7µl)



7: chromatogram of β-sitosterol(10µl

8:Chro matogram of β-sitosterol(12µl)



Track 9:Chromatogram of β-sitosterol(15µl)

Figure 7 : 3D display of β-sitosterol and EASG &CHSG

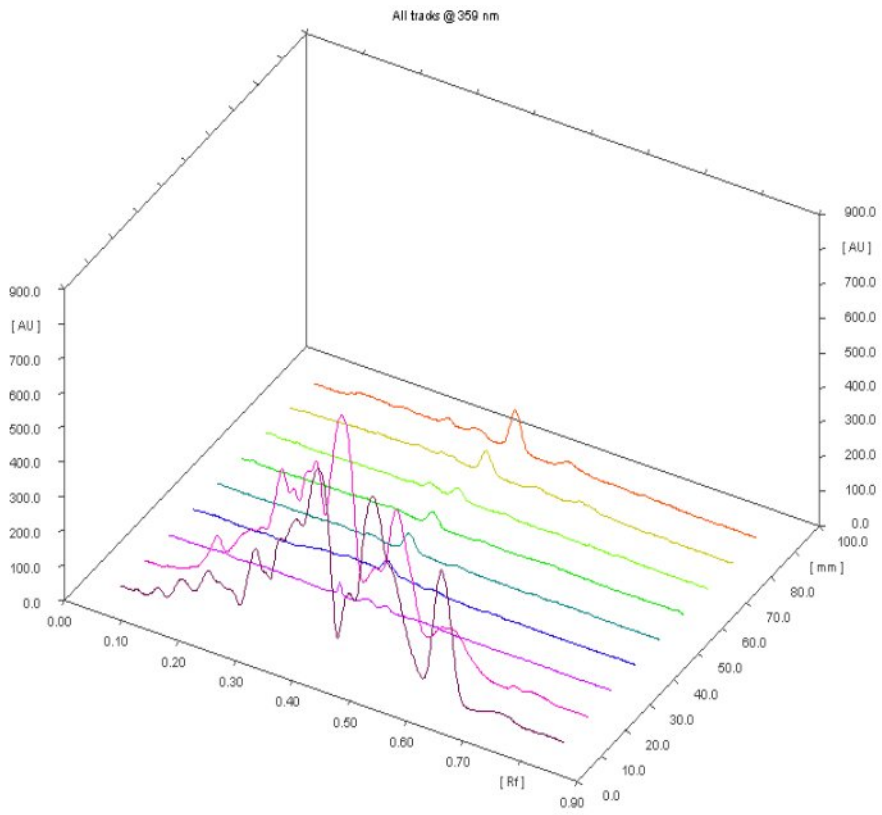


Figure 8: Calibration curve of β-sitosterol

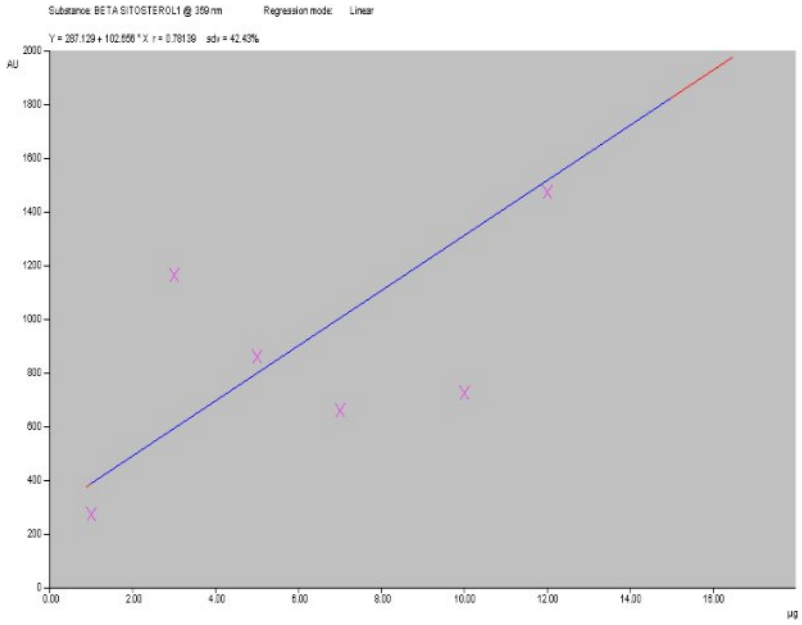


Table 9: Responses obtained for β-sitosterol in preparation of calibration curve for HPTLC studies

Track	R_f	Volume applied (µl)	Area	Remark
3	0.40	1	314.31	β-sitosterol
4	0.40	3	1198.94	β-sitosterol
5	0.39	5	943.97	β-sitosterol
6	0.39	7	727.42	β-sitosterol
7	0.39	10	769.50	β-sitosterol
8	0.40	12	1537.52	β-sitosterol
9	0.41	15	2520.62	β-sitosterol

Table 10: Quantification of β-sitosterol in CHSG & EASG sample by HPTLC

Volume applied (µl)	Area	Amount of β-sitosterol present(µg)	% of β-sitosterol In 100 mg/g 0f extract
(EASG extract)10µl	16849.49	42.16	4.21
(CHSG extract)10µl	20538.19	51.39	5.13

4.4. QUANTIFICATION OF TOTAL PHENOL AND FLAVANOIDS.

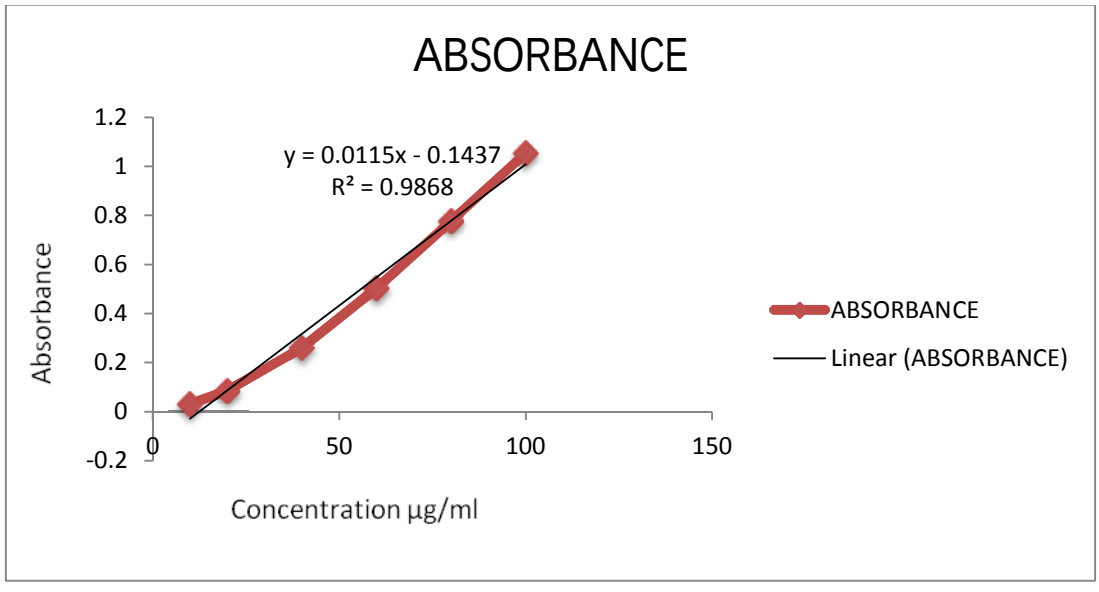
4.4.1. ESTIMATION OF TOTAL PHENOL

Table 11: Estimation of total phenolic content of CHSG & EASG.

Sl.no	Sample	Concentration(µg/ml)	Absorbance
1	Gallic acid 1mg/ml (standard)	10	0.0498
		20	0.0971
		40	0.1907
		60	0.2983
		80	0.410
		100	0.5086
2	CHSG & EASG 1mg/ml(sample)	Chloroform-1000	1.4928
		Ethylacetate-1000	1.1589

The total phenolic content for EASG extract was found to be 118.35mg/g whereas CHSG extract was found to be 148.709 mg/g calculated as gallic acid equivalent.

Figure 9: Estimation of total phenolic content of CHSG & EASG



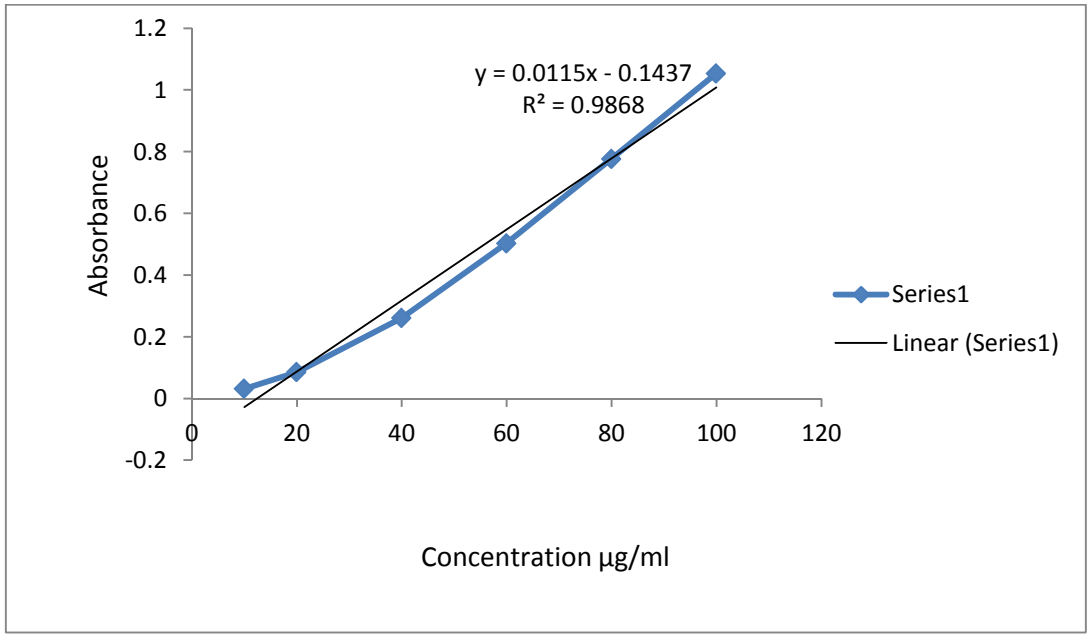
4.4.2.ESTIMATION OF TOTAL FLAVONOID CONTENT.

Table 12: Quantification of Total Flavanoid Content of EASG & CHSG

Sl.No	Sample	Concentration	Absorbance
1	Quercetin 1mg/ml (standard)	10	0.031
		20	0.085
		40	0.26
		60	0.5026
		80	0.776
		100	1.053
2	CHSG &EASG 1mg/ml (sample	Chloroform-1000	0.9701
		Ethylacetate-1000	0.7046

The total flavanoid content of EASG extract was found to be 77.05mg/g whereas CHSG extract was found to be 101.19mg/g calculated as quercetin equivalent.

Figure 10: Estimation of total flavonoid content of CHSG & EASG



4.5. *IN VITRO* ANTIOXIDANT ACTIVITY STUDY IN EASG & CHSG.

4.5.1.DPPH RADICAL SCAVENGING ACTIVITY

Table 13: % inhibition and IC50 values of DPPH radical by ascorbic acid

Sl.No	Concentration (µg/ml)	% inhibition	IC 50 (µg/ml)
1	5	66.58	2.989
2	10	74.34	
3	15	80.58	
4	20	87.48	
5	25	94.56	
6	30	99.38	

Figure 11:DPPH radical scavenging activity of Ascorbic acid

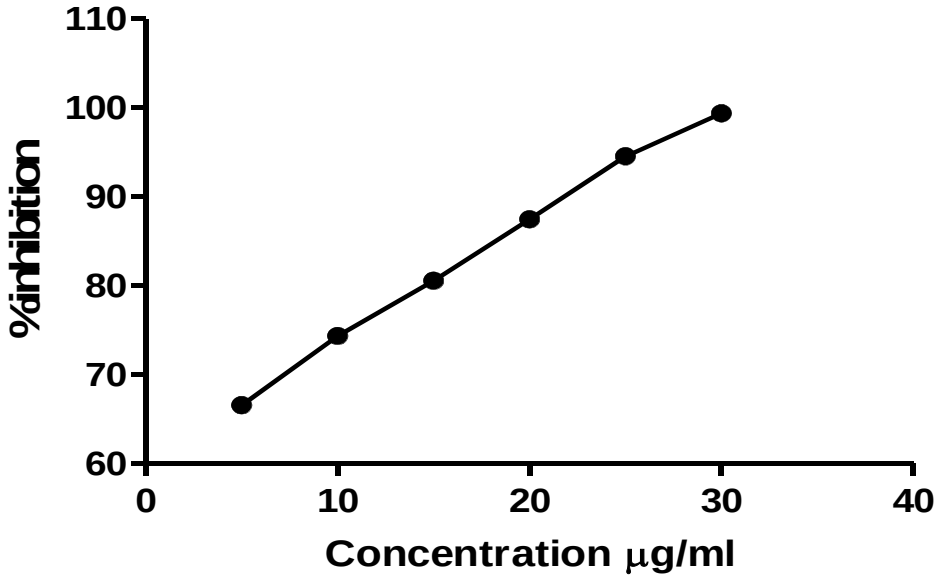


Table 14 : % inhibition and IC50 values of DPPH radical by CHSG

Sl.No	Concentration (µg/ml)	%inhibition	IC_{50} (µg/ml)
1	10	48.29	11.94
2	15	50.23	
3	20	56.20	
4	25	62.04	
5	50	68.28	
6	100	73.19	

Figure 12:DPPH radical scavenging activity of CHSG

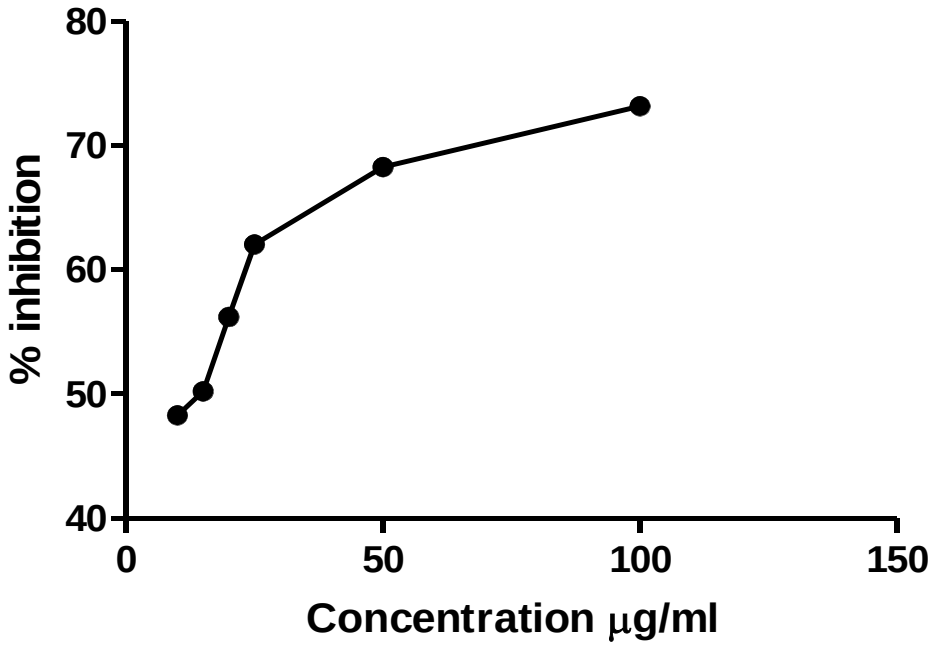
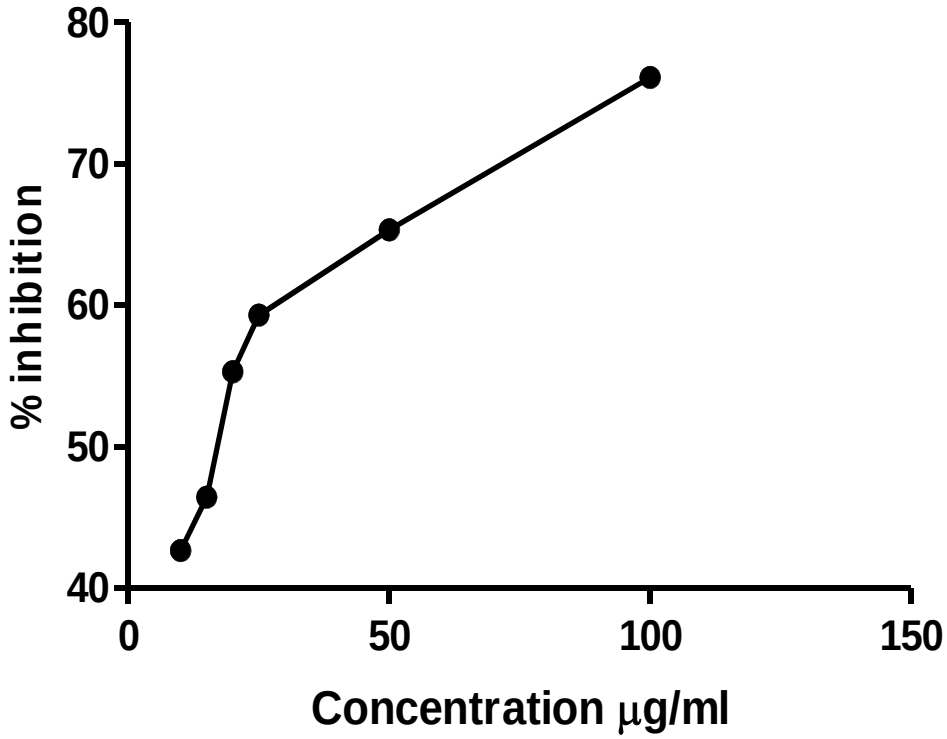


Table 15 : % inhibition and IC50 values of DPPH radical by EASG

Sl.No	Concentration(µg/ml)	% inhibition	IC_{50}
1	10	42.67	15.97
2	15	46.45	
3	20	55.31	
4	25	59.32	
5	50	65.36	
6	100	76.12	

Figure 13: DPPH radical scavenging activity of EASG



4.5.2. TOTAL ANTIOXIDANT ACTIVITY BY ABTS RADICAL CATION ASSAY IN EASG & CHSG.

Table 16: % inhibition of ABTS radical by Ascorbic acid.

Sl.No	Concentration(µg/ml)	%inhibition	IC_{50}
1	0.25	70.08	0.1142
2	0.5	75.22	
3	0.75	79.62	
4	1	85.88	
5	1.25	91.9	
6	1.5	98.85	

Figure 14: ABTS radical scavenging activity of Ascorbic acid

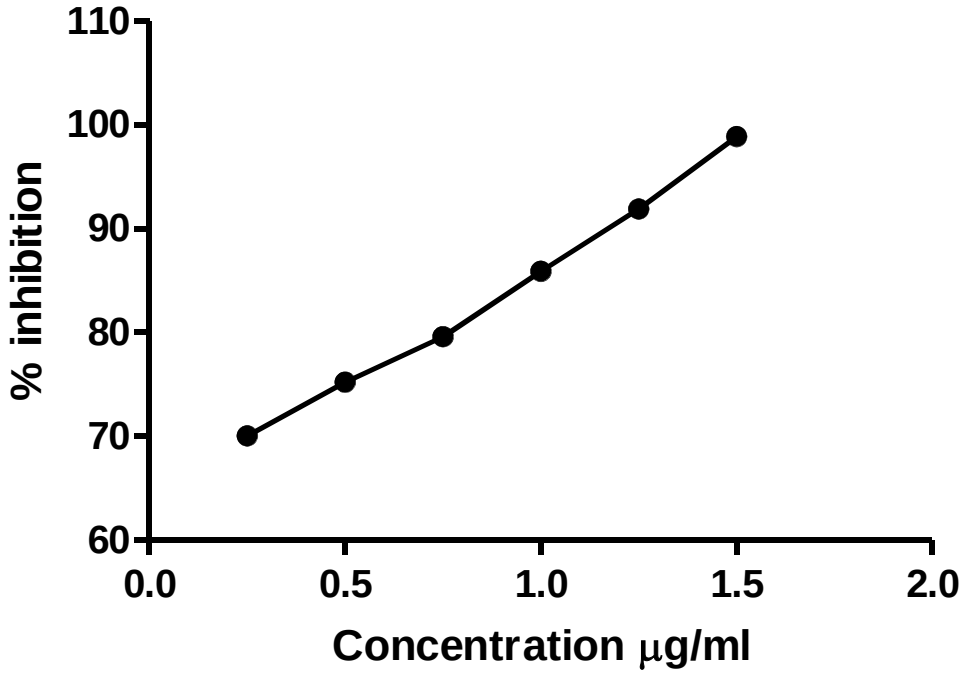


Table 17: % inhibition and IC50 values of ABTS radical by EASG

Sl.No	Concentration (µg/ml)	% inhibition	IC_{50}
1	1	53.61	1.160
2	2	57.41	
3	3	61.63	
4	4	71.18	
5	5	76.92	
6	10	97.37	

Figure 15: ABTS radical scavenging activity of EASG

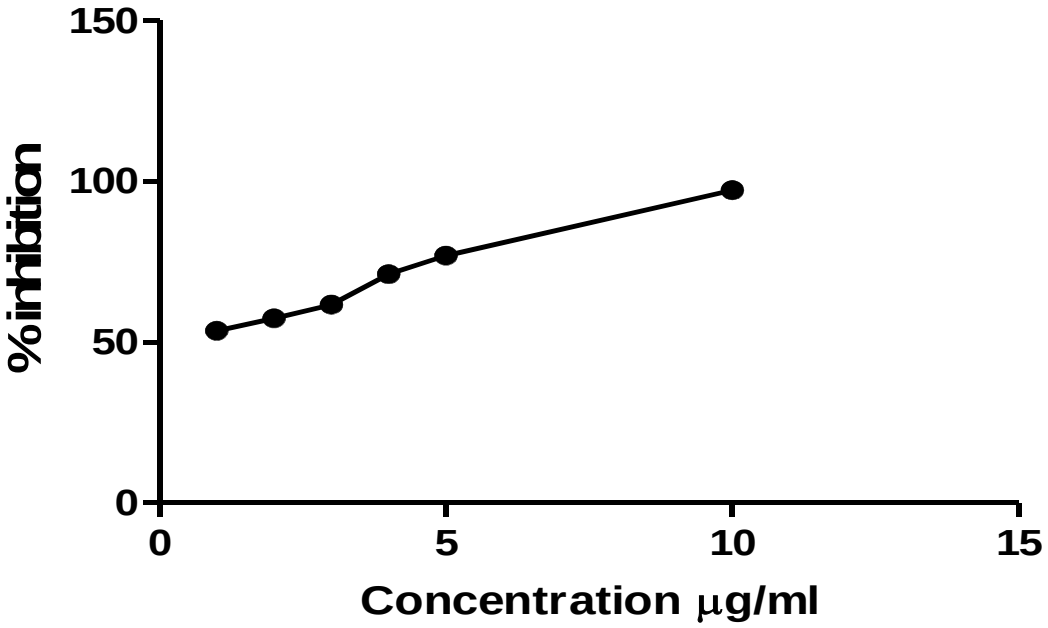
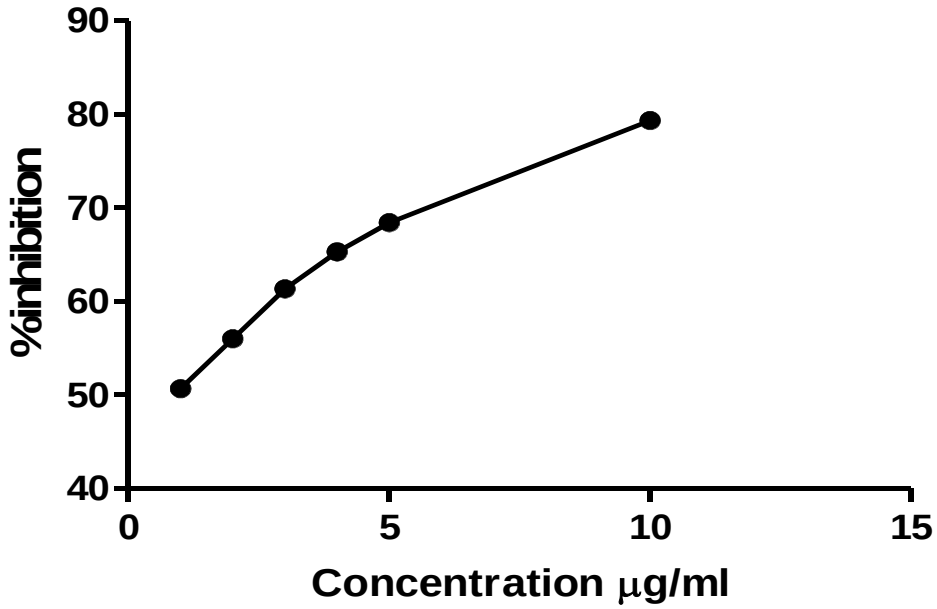


Table 18: % inhibition and IC50 values of ABTS radical by CHSG

Sl.No	Concentration(µg/ml)	% inhibition	IC_{50} (µg/ml)
1	1	50.67	1.134
2	2	56.04	
3	3	61.34	
4	4	65.32	
5	5	68.45	
6	10	79.36	

Figure 16: ABTS radical scavenging activity of CHSG



4.6. INVITRO CYTOTOXICITY STUDY OF EASG & CHSG.

4.6.1. MTT assay on CaCO2 with EASG extract.

Table 19: Cytotoxic properties of EASG against MTT assay on CaCO2 .

Sl.no	Name of the test sample	Test Conc. (µg/ml)	% cytotoxicity	CTC_{50} (µg/ml)
1	EASG extract(1mg/ml)	62.5 125 250 500 1000	8.67±4.6 13.35±1.1 19.01±2.3 25.90±2.1 57.38±0.6	883.33±5.8

Figure 17: cytotoxicity properties of EASG extract against MTT assay on caco2.

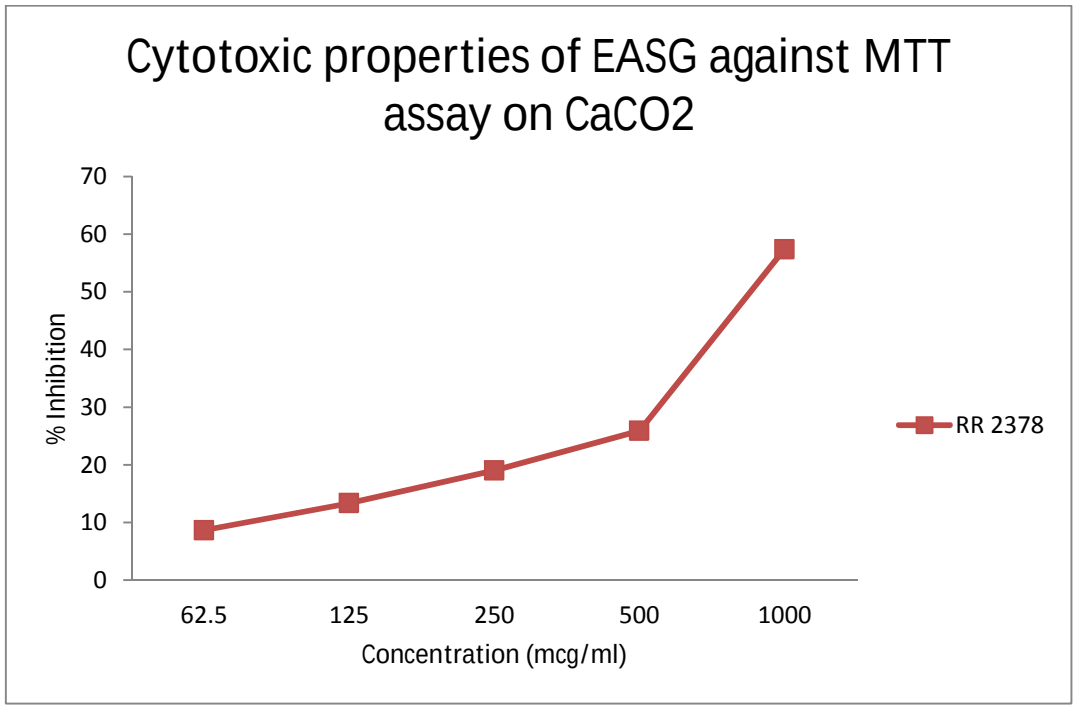
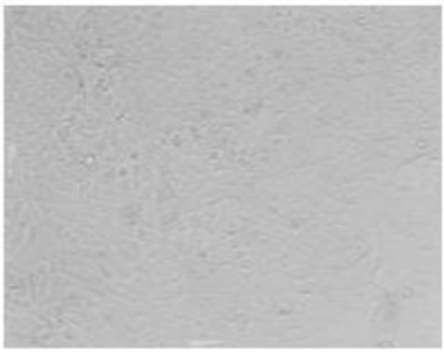
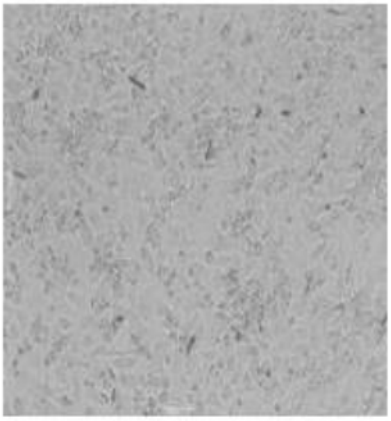


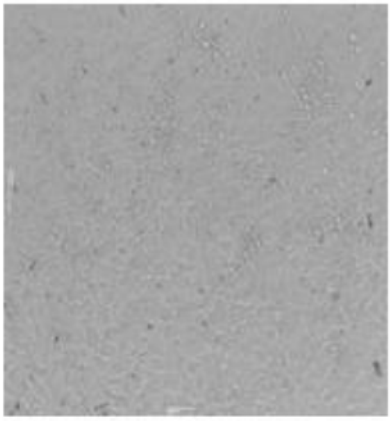
Figure 18: Representative photo micrograph shows morphological changes of Caco2 cell lines with EASG extract.



CONTROL CaCo2



EASG extract 500 (µg/ml)



EASG extract 1000 (µg/ml)

4.6.2. MTT assay on CaCO2 with CHSG Extract.

Table 20: % cytotoxicity properties of CHSG against MTT assay on CaCo2.

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% cytotoxicity	CTC_{50} (µg/ml)
2	CHSG extract(1mg/ml)	62.5 125 250 500 1000	5.04±4.8 6.53±4.8 13.13±3.2 17.37±2.6 24.48±1.3	>1000

Figure 19: cytotoxicity properties of CHSG extract against MTT assay on caco2

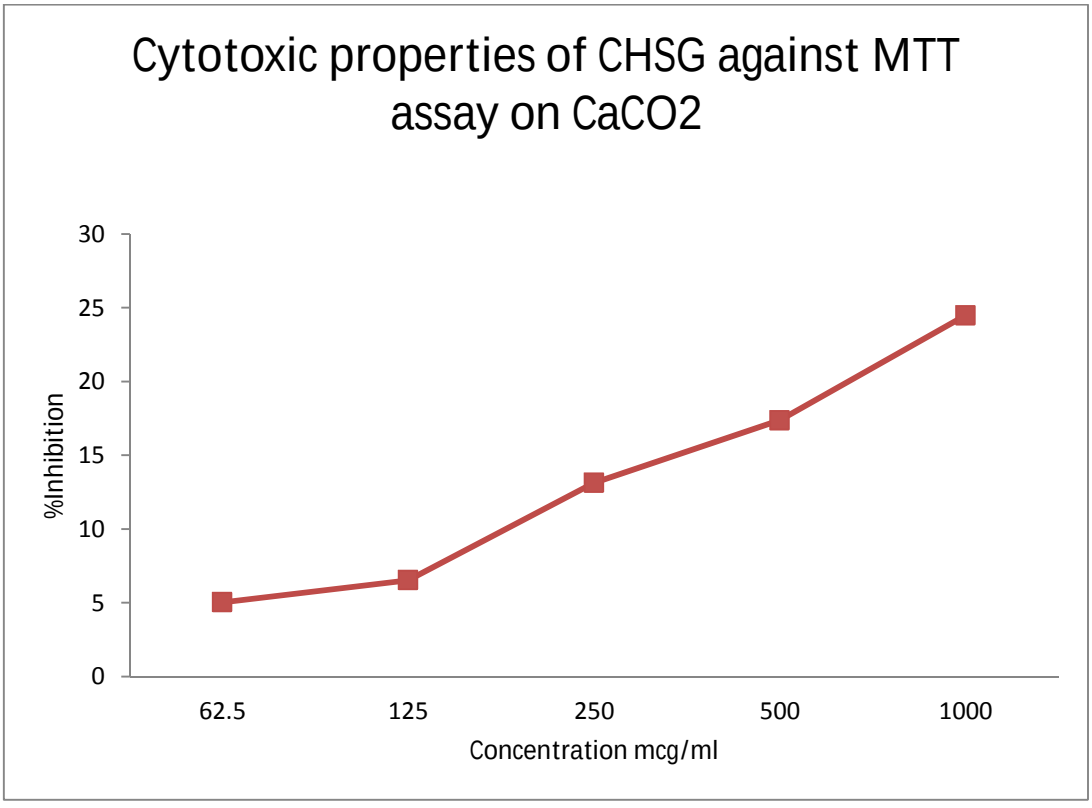
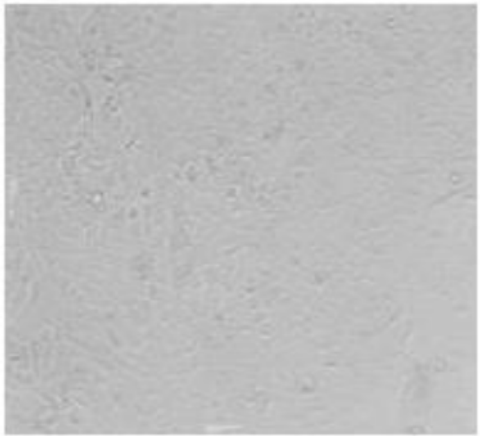
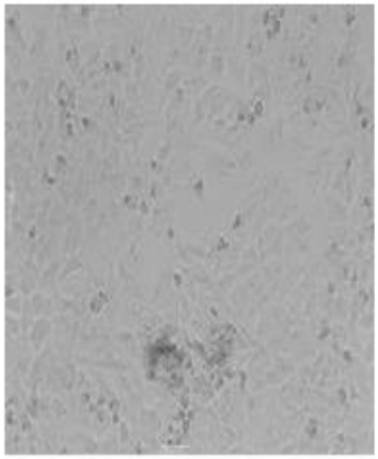


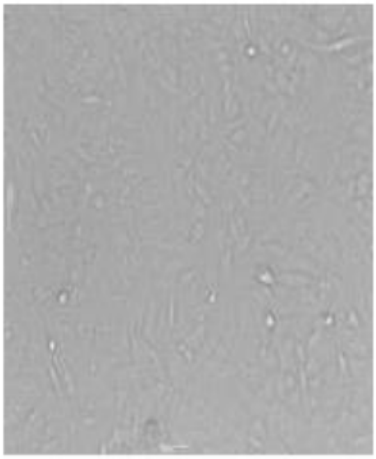
Figure 20: representative photo micrograph shows morphological changes of Caco2 cell lines with CHSG extract.



CONTROL CaCo2



CHSG extract 500 (µg/ml)



CHSG extract1000 (µg/ml)

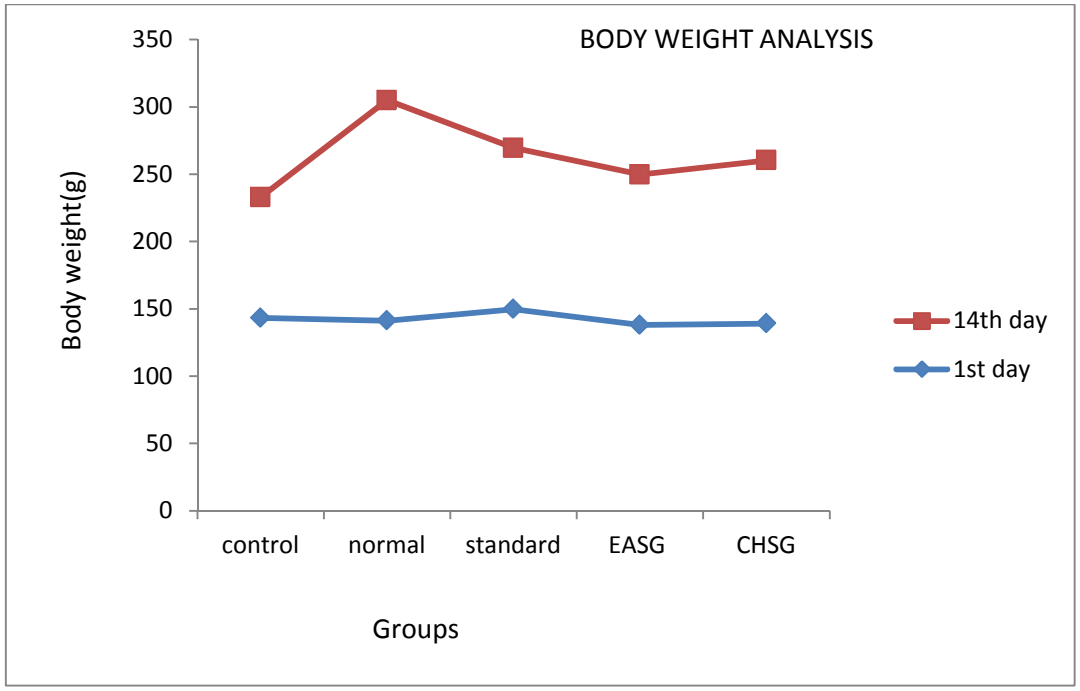
4.7. EVALUATION OF INFLAMMATORY BOWEL DISEASE OF CHSG& EASG.

4.7.1.INDOMETHACIN-INDUCED ENTEROCOLITIS .

Table 21:Evaluation based on bodyweight in indomethacin induced enterocolitis

Groups	1st day body weight	14th day body weight
Group-1normal	141.2±5.069	163.7±4.402
Group-II control	143±7.956	89.67±2.692***
GROUP-IIIStandard	149±16.06	119.7±8.086***
GROUP-IVEASG treated	138.0±7.412	111.7±4.201*
GROUP-VCHSG treated	139.0±4.211	121.3±3.836***

Figure 21:Body weight analysis



Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed. $^{a}P<0.001$ denotes comparison of Inflammatory bowel disease control with vehicle control and ns- non significant $*P<0.05$, $**P<0.01$, and $***P<0.001$ denotes comparison of all groups with Inflammatory bowel disease control

Table 22: Evaluation based on macroscopic feature in Indomethacin- induced enterocolitis.

Group	**Treatment**	**Mean of macroscopic scores ± S.E.M**
Group I: normal	Only normal saline	0.0±0.0
Group II: control	Indomethacin (7.5mg/kg,s.c)	5.667±0.1520***
Group III: Standard	Prednisolone(10mg/kg,orally) and indomethacin(7.5mg/kg))	1.000±0.2191***
GroupIV:EASG(400mg/kg)	Ethyl acetate extract of *simarouba glauca* (400mg/kg) and indomethacin (7.5mg/kg)	1.133±0.2765***
GroupV:CHSG (400mg/kg)	Chloroform extract of *Simarouba glauca* (400mg/kg,orally) and indomethacin (7.5mg/kg	2.200±0.4099***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed. $^{a}P<0.001$ denotes comparison of Inflammatory bowel disease control with vehicle control and ns- non significant $*P<0.05$, $**P<0.01$, and $***P<0.001$ denotes comparison of all groups with Inflammatory bowel disease control

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Figure 22 :Effect of EASG & CHSG extract on Macroscopic features in indomethacin induced enterocolitis

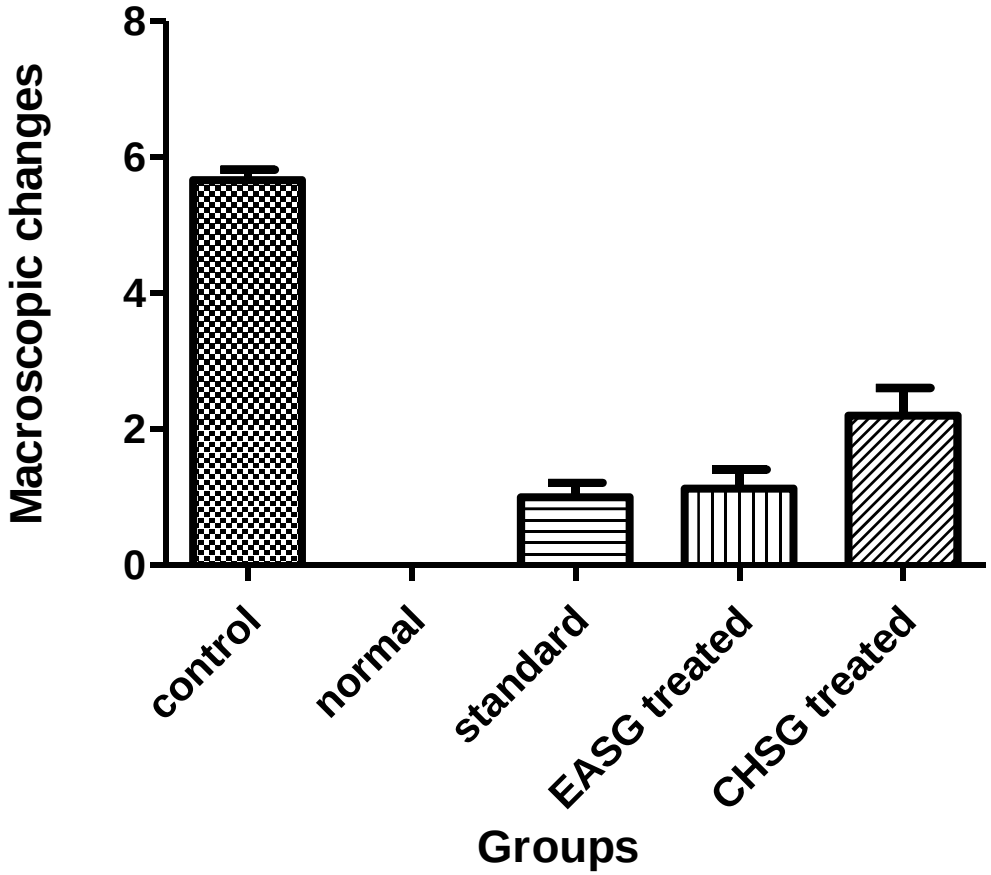
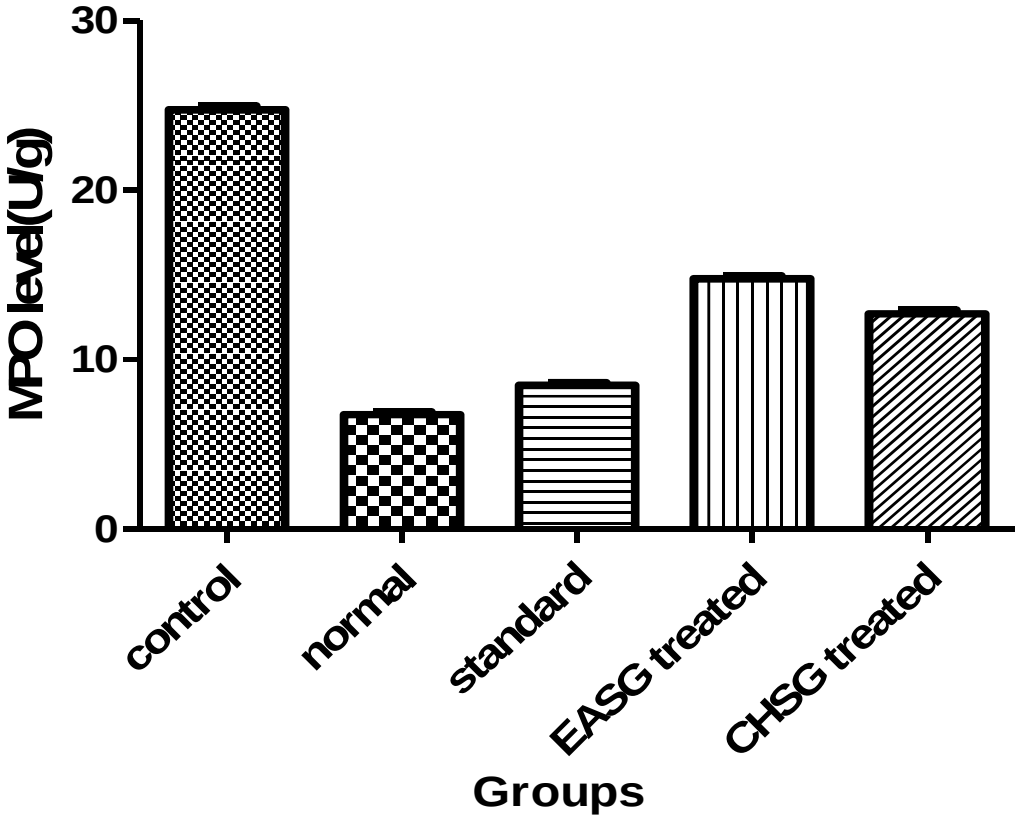


Table 23: Evaluation based on MPO activity in Indomethacin-induced enterocolitis.

Group	**Treatment**	**MPO activity(U/g)± S.E.M**
Group I: normal	Only normal saline	6.733±0.1695
Group II: control	Indomethacin (7.5mg/kg,s.c)	24.90±0.2769***
Group III: Standard	Prednisolone(10mg/kg,orally) and indomethacin(7.5mg/kg)	8.473±0.1314***
GroupIV:EASG(400mg/kg)	Ethyl acetate extract of *simarouba glauca* (400mg/kg) and indomethacin (7.5mg/kg)	14.77±0.1520***
GroupV:CHSG (400mg/kg)	Chloroform extract of *simarouba glauca* (400mg/kg,orally) and indomethacin (7.5mg/kg	12.69±0.2143***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed. $^{a}P<0.001$ denotes comparison of Inflammatory bowel disease control with vehicle control and ns- non significant $*P<0.05$, $**P<0.01$, and $***P<0.001$ denotes comparison of all groups with Inflammatory bowel disease control.

Figure 23: Effect of EASG & CHSG extract on MPO activity in indomethacin-induced enterocolitis



4.7.1.1. INTESTINAL IMAGES OF INDOMETHACIN INDUCED ENTEROCOLITIS

Figure 24: Images of control group(untreated)

Duodenum Jejunum ileum Caecum Colon

Figure 25: Images of normal group

Duodenum jejunum ileum Caecum colon

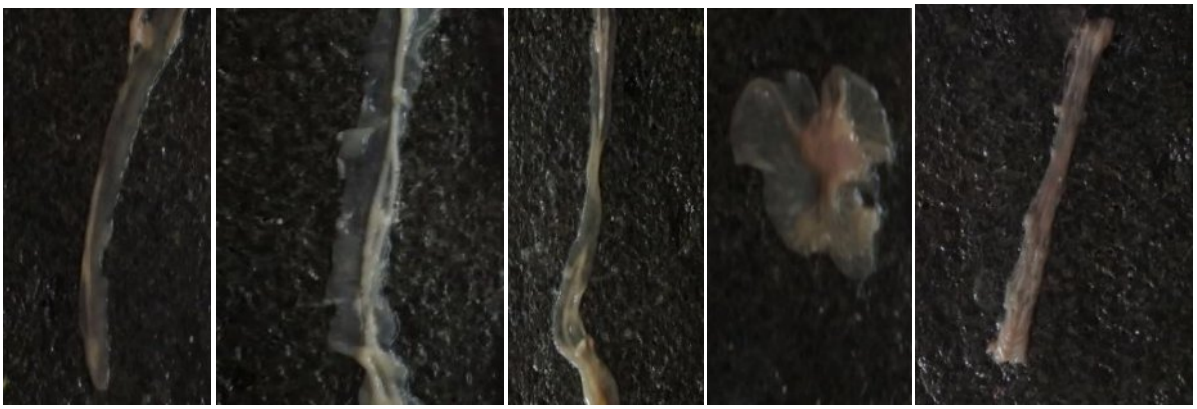
Figure 26: Images of standard treated group

Duodenum jejunum ileum Caecum Colon

Figure27: Images of ethyl acetate extract treated group

Duodenum jejunum ileum caecum Colon

Figure 28: Images of chloroform extract treated group



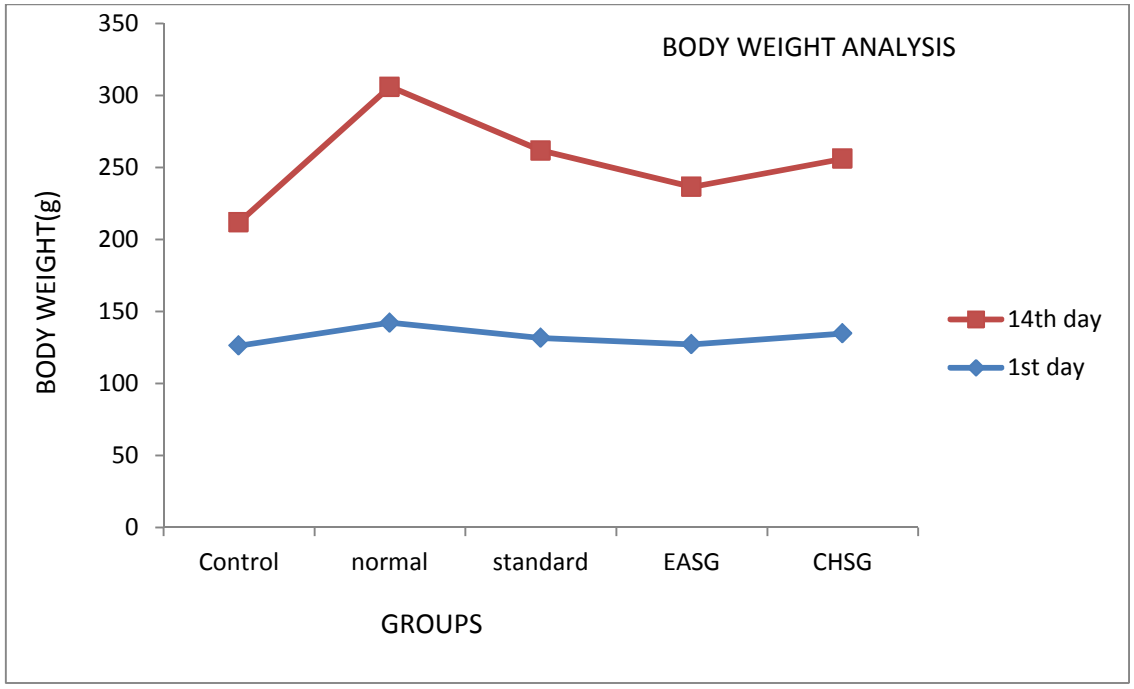
Duodenum jejunum ileum caecum colon

4.7.2.ACETIC ACID-INDUCED ENTEROCOLITIS .

Table 24:Evaluation based on bodyweight in acetic induced enterocolitis.

Groups	1st day body weight	14th day body weight
GroupI-control	126.3±4.177	85.50±3.775***
GroupII-normal	142.2±5.534	163.7±4.402
GroupIII-Standard	131.7±4.595	130.0±3.907***
GroupIV-EASG treated	127.2±5.724	109.3±4.645**
GroupVCHSG treated	134.8±4.556	121.8±5.023***

Figure 29: Body weight analysis of acetic acid induced enterocolitis



Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One wayAnnova followed by Dunnett's test was performed. *aP<0.001* denotes comparison of inflammatory bowel disease control with vehicle control and ns- non significant **P<0.05*, ***P<0.01*, and ****P<0.001* denotes comparison of all groups with Inflammatory Bowel disease control.

Table 25: Evaluation based on macroscopic features in acetic acid-induced enterocolitis.

Groups	**Treatment**	**Mean of macroscopic scores ± S.E.M**
Group I: normal	Only normal saline	0.0±0.0
Group II: control	6% acetic acid solution,intrarectally	3.667±0.5426***
Group III: Standard	Prednisolone(10mg/kg,orally) and 6% acetic acid solution,intrarectally	0.7500±0.2419***
GroupIV:EASG(400mg/kg)	Ethyl acetate extract of *Simarouba glauca* (400mg/kg) and 6% acetic acid solution,intrarectally	2.500±0.1826*
GroupV:CHSG (400mg/kg)	Chloroform extract of *Simarouba glauca* (400mg/kg,orally) and 6% acetic acid solution intrarectally.	1.917±0.3005**

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One wayAnnova followed by Dunnett's test was performed. *aP<0.001* denotes comparison of inflammatory bowel disease control with vehicle control and ns- non significant **P<0.05*, ***P<0.01*, and ****P<0.001* denotes comparison of all groups with Inflammatory Bowel disease control.

Figure 30: Effect of EASG and CHSG on macroscopic features in acetic-acid induced enterocolitis

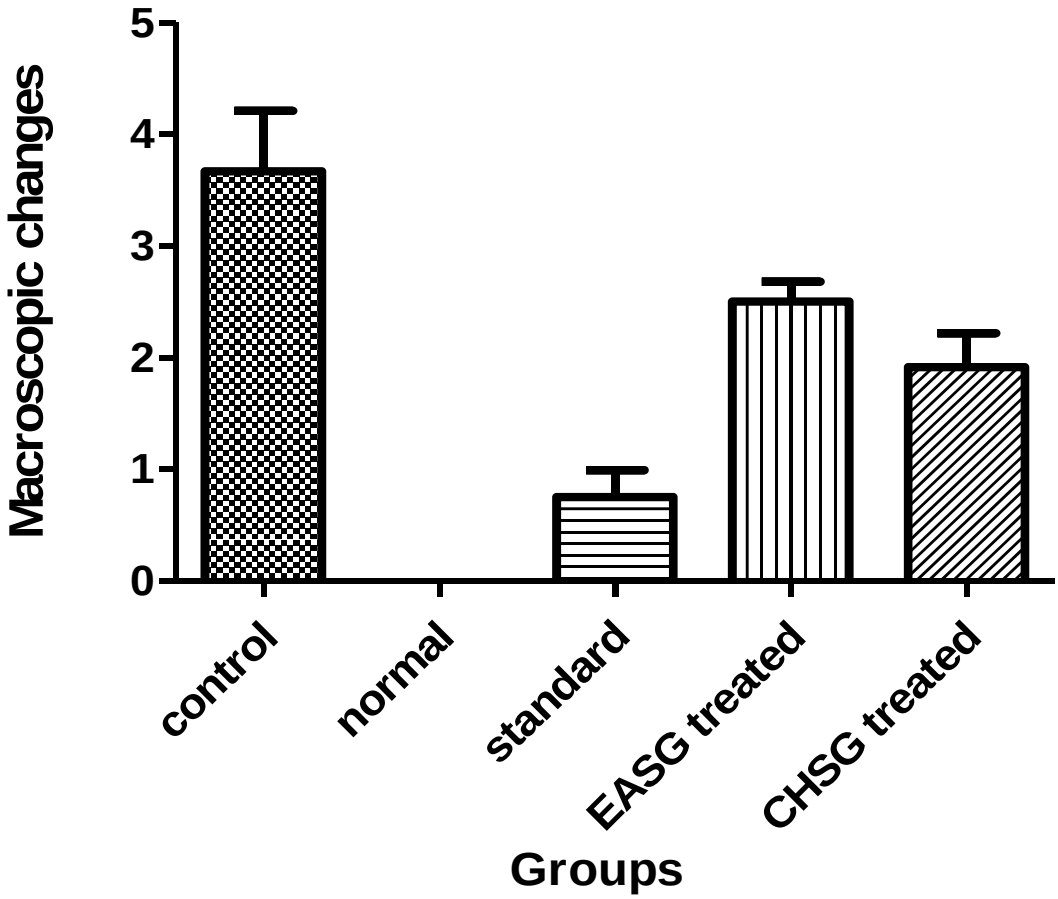
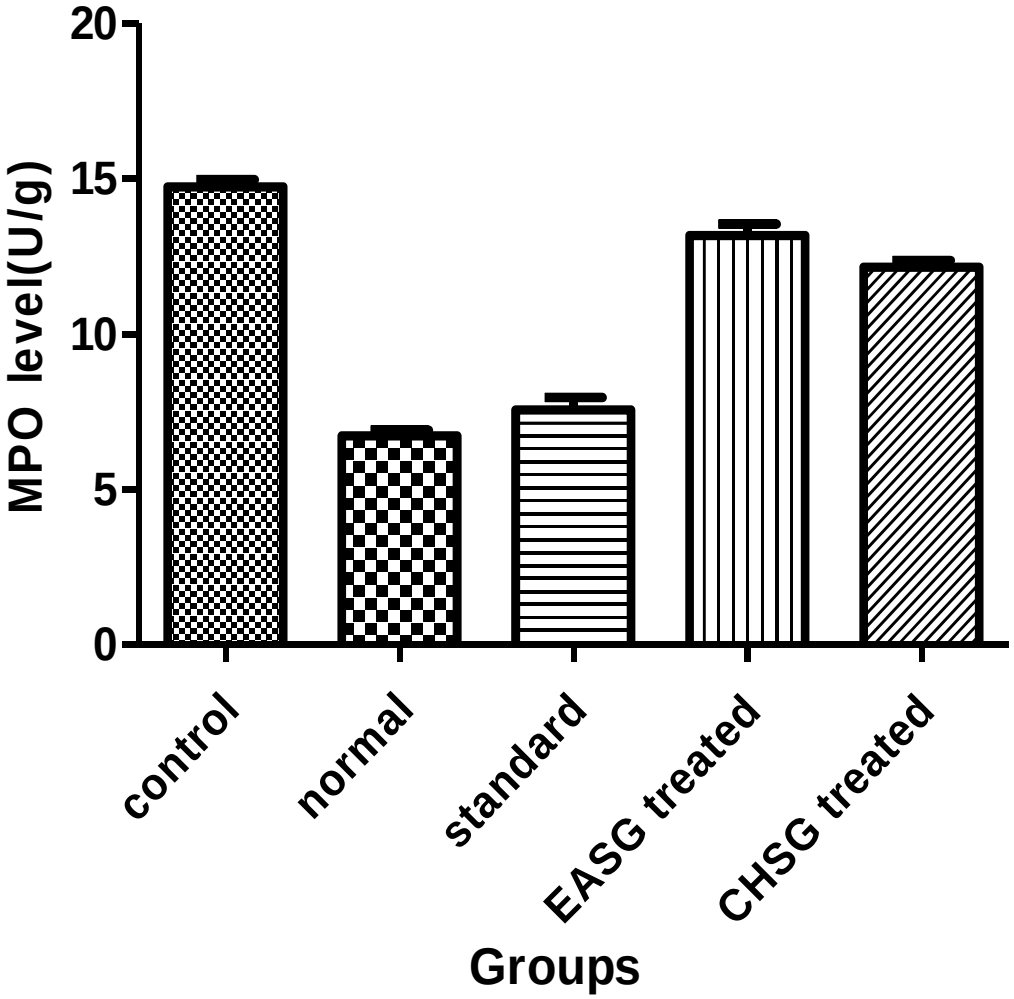


Table 26: Evaluation of MPO activity in acetic acid-induced enterocolitis.

Group	Treatment	MPO activity(U/g)± S.E.M
Group I: normal	Only normal saline	6.733±0.1695
Group II: control	6% acetic acid solution,intrarectally	14.74±0.2365***
Group III: Standard	Prednisolone(10mg/kg,orally) and 6% acetic acid solution,intrarectally	7.564±0.3992***
GroupIV:EASG(400mg/kg)	Ethyl acetate extract of *simarouba glauca* (400mg/kg) and 6% acetic acid solution,intrarectally	13.18±0.3600**
GroupV:CHSG (400mg/kg)	Chloroform extract of *simarouba glauca* (400mg/kg,orally) and 6% acetic acid solution intrarectally.	12.15±0.2311***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One wayAnnova followed by Dunnett's test was performed. *aP<0.001* denotes comparison of inflammatory bowel disease control with vehicle control and ns-non significant **P<0.05*, ***P<0.01*, and ****P<0.001* denotes comparison of all groups with Inflammatory Bowel disease control.

Figure 31: Effect of EASG & CHSG on MPO in acetic acid induced enterocolitis



4.7.2.1.IMAGES OF CAECUM AND COLON IN ACETIC ACID INDUCED ENTEROCOLITIS

Figure 32:Images of control group

Caecum Colon

Figure 33: Images of standard treated group

Colon **Caecum**

Figure 34:Images of ethyl acetate extract treated group

Colon **Caecum**

Figure 35: Images of chloroform extract treated group

Colon **Caecum**

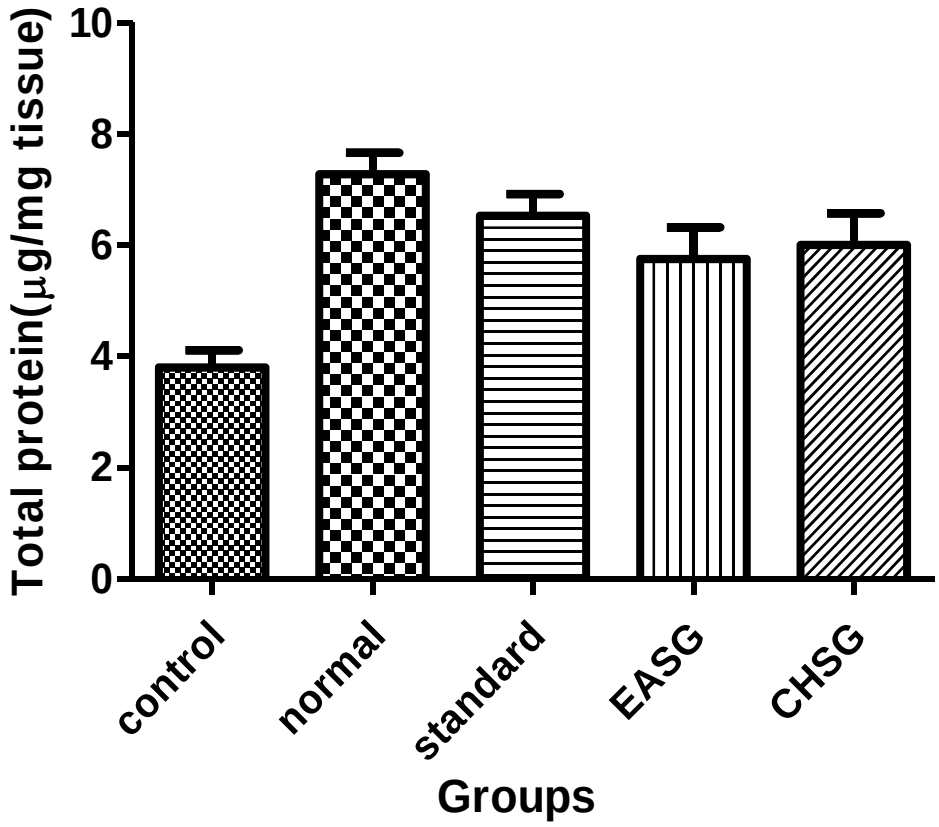
4.8.ESTIMATION OF TOTAL PROTEIN

Table 27: Effect of CHSG &EASG on total protein in intestinal tissue of indomethacin induced enterocolitis

Groups	Total protein (mg/100mg of tissue)
GroupI-normal	7.282±0.3772
GroupII-control	3.805±0.3036***
Group III-Standard	6.528±5.753**
Group IV-CHSG(400mg/kg)	6.005±0.5716**
Group V-EASG(400mg/kg)	5.753±0.5709*

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One wayAnnova followed by Dunnett's test was performed. *aP<0.001* denotes comparison of inflammatory bowel disease control with vehicle control and ns-non significant **P<0.05*, ***P<0.01*, and ****P<0.001* denotes comparison of all groups with Inflammatory Bowel disease control.

Figure 36: Effect of EASG and CHSG extract on total protein in indomethacin induced enterocolitis



4.9. *INVIVO* ANTIOXIDANT ACTIVITY

Table 28: Effect of CHSG &EASG on Invivo antioxidants of intestinal tissues of indomethacin-induced enterocolitis in rats.

Groups	SOD(unit/min/ mg protein)	CAT (µmol of H2O2 consumed/ min/ mg protein)	GSH (Glutathione µg/mg)	GPx(nmol of glutathione oxidized/min/ mg protein)	LPO (nmol of MDA/mgprotein
Control	1.920±0.2487***	15.48±0.5160***	8.452±0.3487***	12.25±0.3374***	21.83±0.7491***
normal	6.988±0.2028	50.02±0.8185	16.39±0.3367	31.25±1.588	8.350±0.3603
Standard	4.965±0.1803***	29.14±0.6283***	13.57±0.2049***	24.40±0.4574***	9.971±0.3147***
EASG treated	3.083±0.2283**	17.78±0.4095*	10.37±0.3495**	15.28±0.4778*	18.67±0.6667**
CHSG treated	3.400±0.2299***	18.87±0.4711**	12.44±0.6641***	16.87±0.4793**	13.83±0.4773***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One wayAnnova followed by Dunnett's test was performed. *aP<0.001* denotes comparison of inflammatory bowel disease control with vehicle control and ns-non significant **P<0.05*, ***P<0.01*, and ****P<0.001* denotes comparison of all groups with Inflammatory Bowel disease control.

Figure 37: Effect of EASG& CHSG on SOD in indomethacin induced enterocolitis

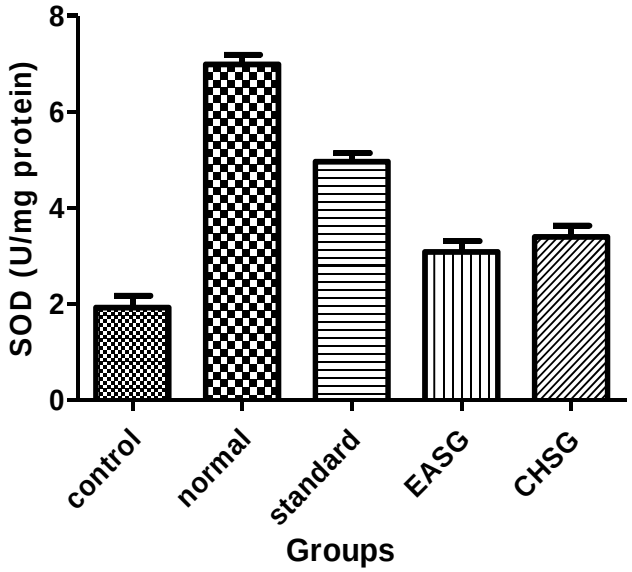


Figure 38: Effect of EASG& CHSG on catalase in indomethacin induced enterocolitis

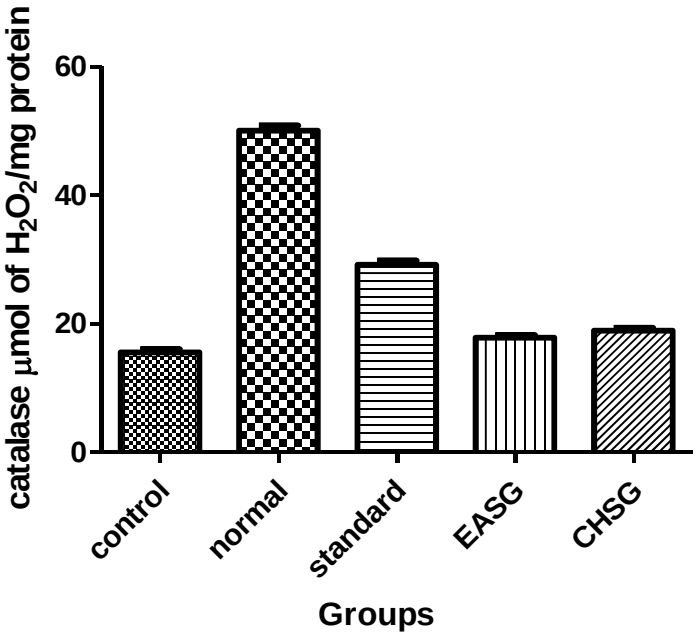


Figure 39: Effect of EASG& CHSG on GSH in indomethacin induced enterocolitis

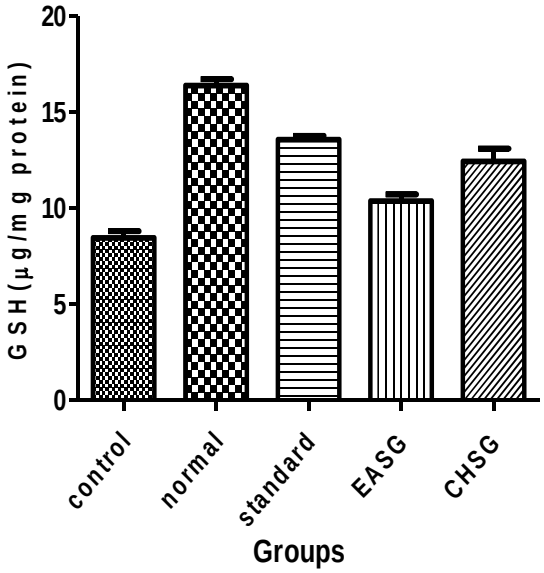


Figure 40: Effect of EASG& CHSG on GPx in indomethacin induced enterocolitis

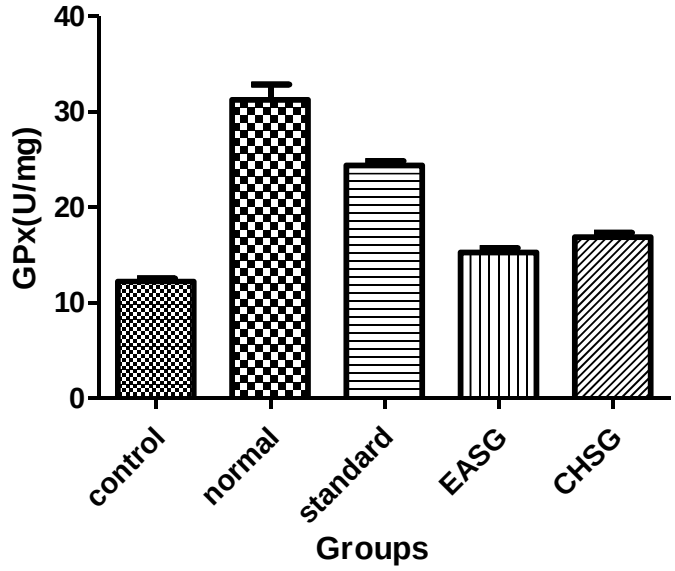


Figure 41: Effect of EASG& CHSG on LPO in indomethacin induced enterocolitis

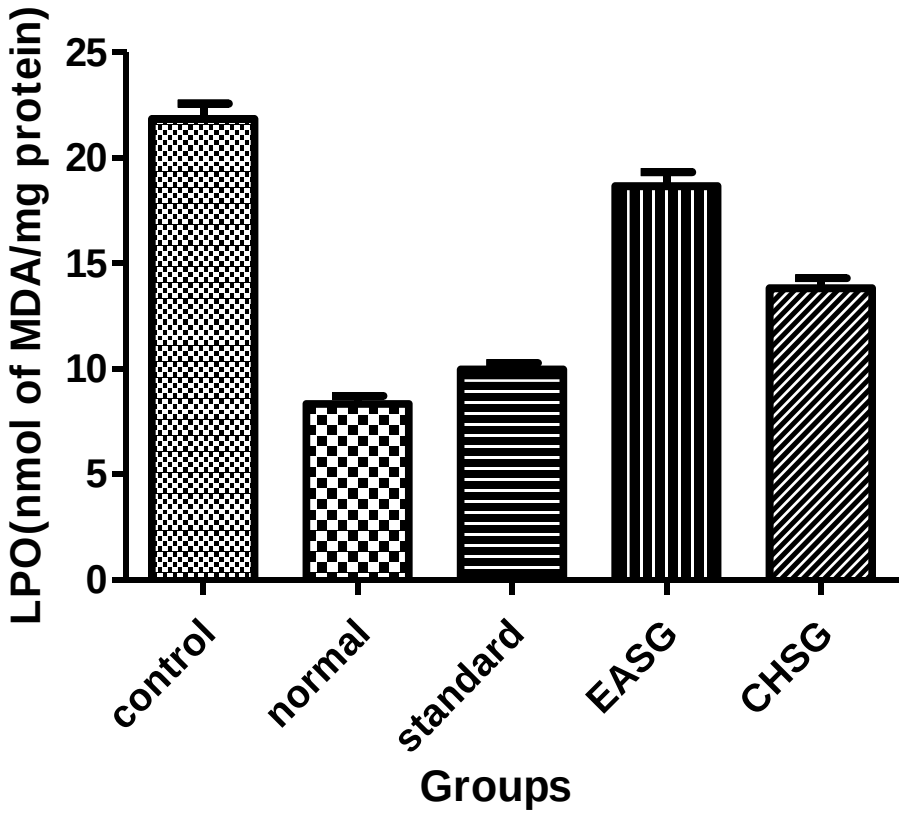


Table 29: Effect of CHSG &EASG on total protein in intestinal tissue of acetic acid induced enterocolitis

Groups	Total protein (mg/100mg of tissue)
GroupI-normal	5.663±0.3288
GroupII-control	1.825±0.2687***
GroupIII-Standard prednisolone(10mg/kg)	5.167±0.2045***
Group IV- CHSG(400mg/kg)	4.058±0.3084***
Group V-EASG(400mg/kg)	3.556±0.2045**

Figure 42: Effect of EASG& CHSG on total protein in acetic-acid induced enterocolitis

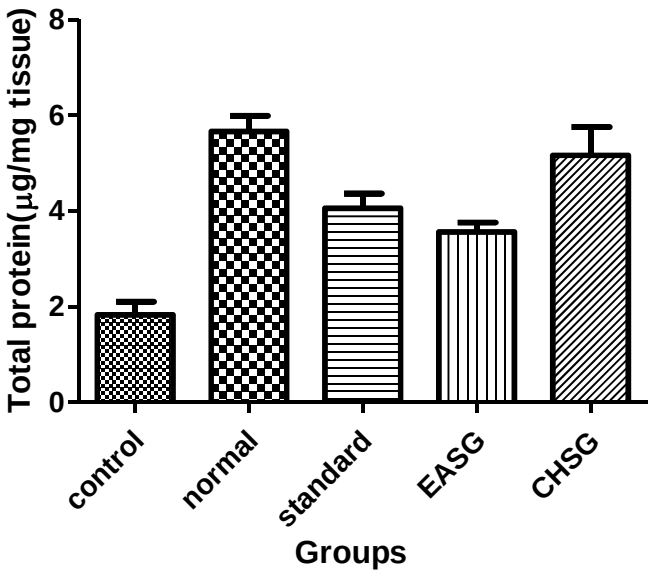


Table 30: Effect of CHSG &EASG on Invivo antioxidants of intestinal tissues of Acetic acid -induced enterocolitis in rats.

Groups	SOD(unit/mg protein)	CAT (µmol of H2O2 consumed/ min/ mg protein)	GSH (Glutathione µg/mg)	GPx(nmol of glutathione oxidized/min/ mg protein)	LPO (nmol of MDA/mg protein
Control	0.9120±0.02899***	12.83±0.2256***	6.742±0.4268**	14.98±1.363***	24.71±0.4423***
normal	4.273±0.2593	64.25±1.225	15.19±0.3236	27.98±0.5553	5.926±0.1193
Standard	3.475±0.1226***	19.16±0.4695***	12.79±0.3493***	22.05±0.3376***	8.108±0.5766***
EASG treated	1.826±0.1855*	16.10±0.5035**	8.329±0.3530*	18.71±0.8231*	21.73±0.7119**
CHSG treated	2.222±0.3515**	17.20±0.5681***	10.87±0.2534***	19.38±0.7954**	16.42±0.5457***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One wayAnnova followed by Dunnett's test was performed. *aP<0.001* denotes comparison of inflammatory bowel disease control with vehicle control and ns-non significant **P<0.05*, ***P<0.01*, and ****P<0.001* denotes comparison of all groups with Inflammatory Bowel disease control.

Figure 43: Effect of EASG& CHSG on SOD inacetic acid induced enterocolitis

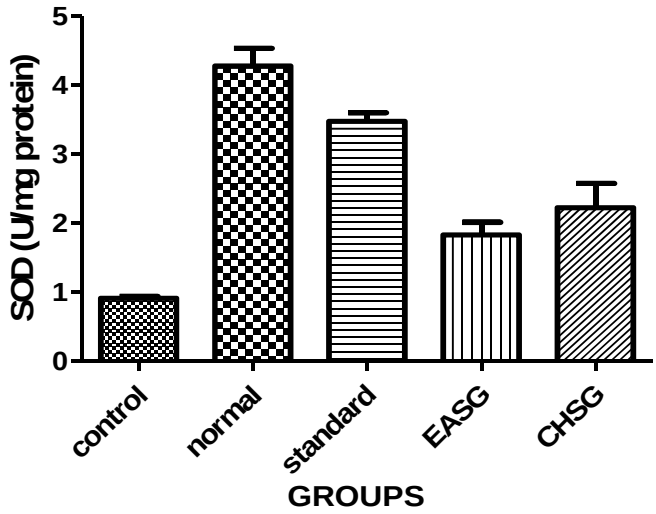


Figure 44: Effect of EASG& CHSG on Catalase in acetic acid induced enterocolitis

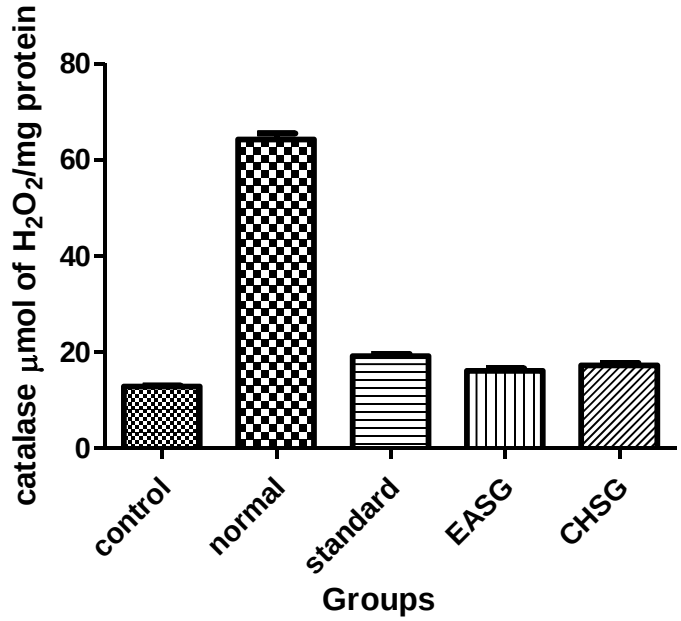


Figure 45: Effect of EASG& CHSG on GSH in acetic acid induced enterocolitis

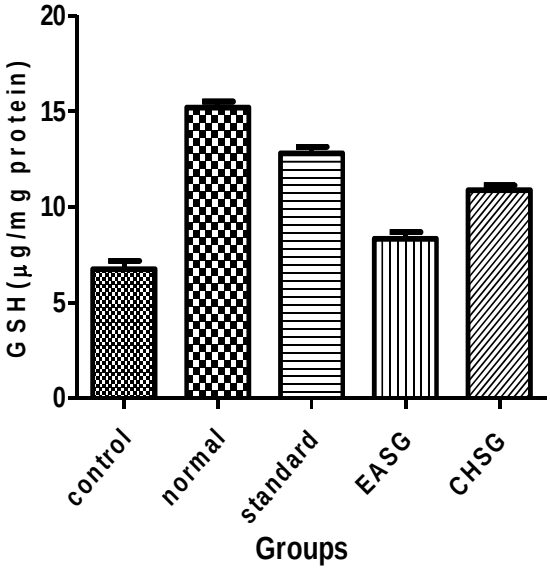


Figure 46: Effect of EASG& CHSG on GPx in acetic acid induced enterocolitis

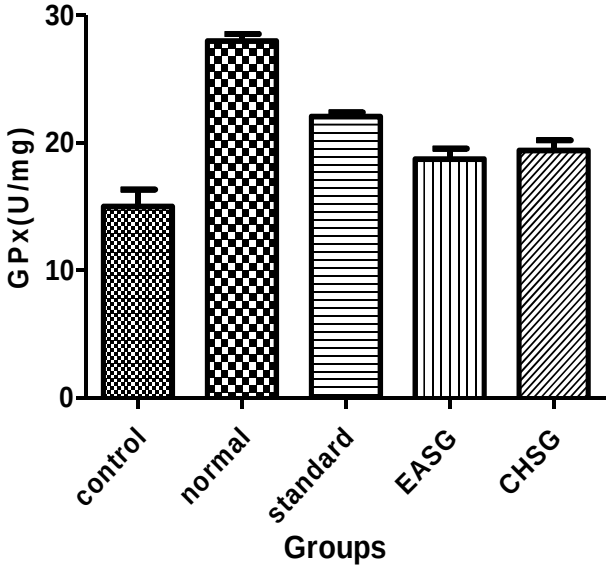
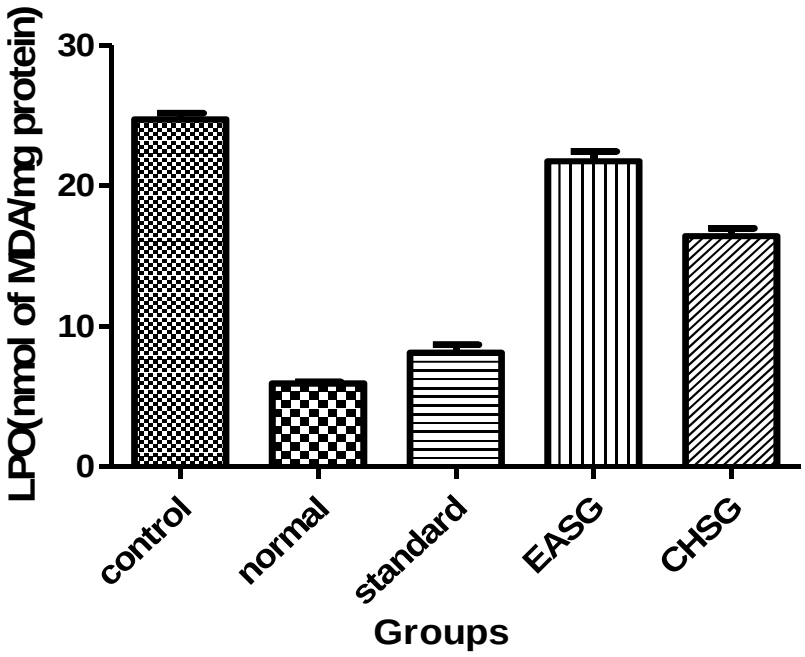


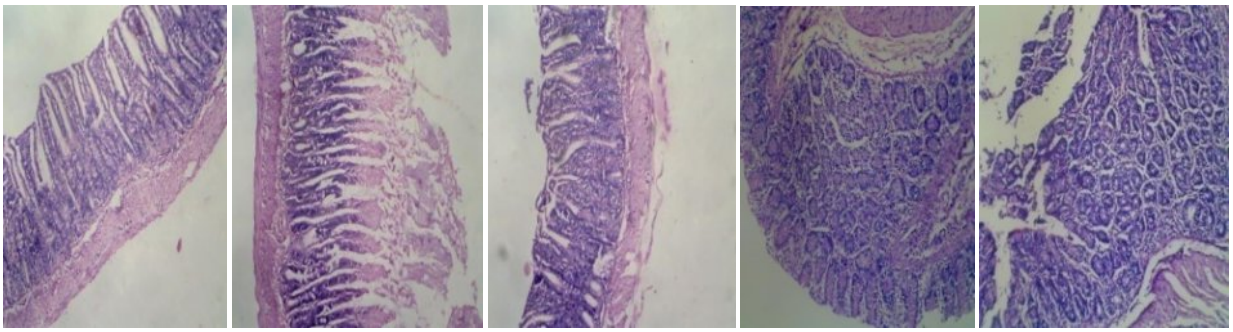
Figure 47: Effect of EASG& CHSG on LPO in acetic acid induced enterocolitis



4.10.HISTOPATHOLOGICAL STUDY

4.10.1.Indomethacin-induced enterocolitis in rats

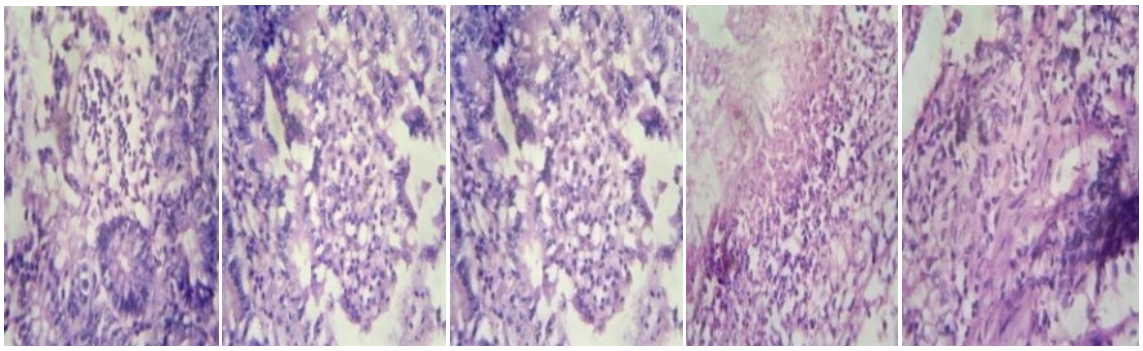
Figure 48 :GROUP-I (normal group)



Duodenum jejunum ileum Caecum colon

- Section from duodenum shows normal epithelium. Lamina propria shows few scattered lymphocytic infiltrates. Submucosa and connective tissues shows no significant pathology. Muscular layer shows normal morphology. There is no malignancy/granuloma/IBD seen in the section studied.
- Section from jejunum shows normal mucosa. Lamina propria shows very mild lymphocytic infiltrates. Muscular layer shows normal. There is no evidence of malignancy/granuloma/IBD seen in the section studied.
- Section from ileum shows normal epithelium. Lamina propria shows few scattered lymphocytic infiltrates. Muscular layer and serosa shows normal morphology. There is no malignancy/granuloma/ IBD seen in the section studied.
- Section from caecum shows normal mucosa.Lamina propria shows normal morphology.Muscular layer shows normal.There is no evidence of cryptitis and crypt abscess.
- Section from colon shows normal epithelium. Lamina propria shows few scattered lymphocytic infiltrates and connective tissues. There is no malignancy/granuloma seen.

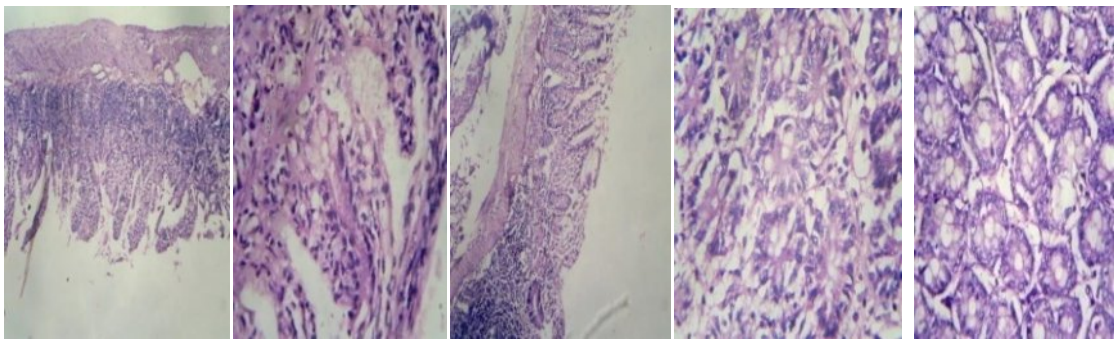
Figure 49: GROUP-II (Indomethacin control group)



Duodenum jejunum ileum caecum colon

- Section from duodenum shows focal surface ulceration. Lamina propria shows lymphoplasmacytic infiltrates. Submucosa shows dilated blood vessels. Muscular layer and serosa shows no significant pathology.
- Section from jejunum shows epithelial ulceration with focal necrosis and edema, surface shows transudates. Lamina propria shows lamina propria shows lymphoplasmacytic infiltrates. Muscular layer and serosa shows normal.
- Section from ileum shows focal surface ulceration. Lamina propria shows moderate lymphocytic infiltrates. Submucosa shows peyer's patches. Muscular layer and serosa shows normal morphology.
- Section from caecum shows full thickness necrosis (gangrene). Lamina propria shows dense inflammatory infiltrates. Muscular layer shows no significant pathology. Serosa shows mild inflammatory infiltrates. Consistent with gangrene.
- Section from colon mucosa shows epithelial ulceration. Lamina propria shows dense lymphocytic infiltrates and few glands shows loss of architecture (cryptitis). Muscular layer and serosa shows no significant pathology.

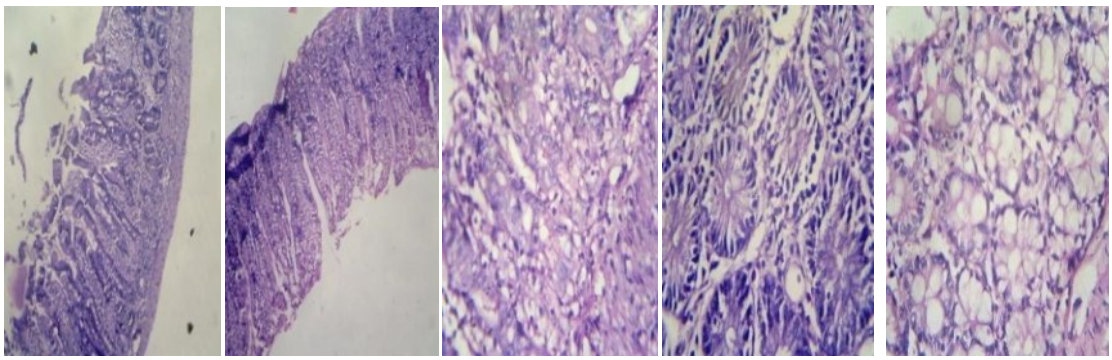
Figure 50: GROUP-III (standard treated group)



Duodenum **jejunum** **ileum** **caecum** **colon**

- Section from duodenum shows epithelial ulceration. Lamina propria shows scattered lymphocytic infiltrates. Submucosa shows dilated blood vessels. Muscular layer and serosa shows no significant pathology
- Section from jejunum shows epithelial ulceration. Lamina propria shows chronic inflammatory infiltrates on focal shows complete loss of glands. Muscular layer and serosa shows normal. There is no evidence of malignancy seen in the section studied.
- Section from ileum shows ulceration in the mucosa. Lamina propria shows few moderate lymphocytic infiltrates. Muscular layer and serosa shows normal morphology.
- Section from caecum shows mucosal ulceration, one focal shows full thickness ulceration. Lamina propria shows dense inflammatory infiltrates with focal areas shows mild cryptitis. Muscular layer shows normal morphology. Serosa shows mild inflammatory infiltrates.
- Section from colon shows mucosal ulceration. Lamina propria shows diffuse less lymphocytic infiltrates and few glands shows loss of architecture (cryptitis). Muscular layer and serosa shows mild inflammation.

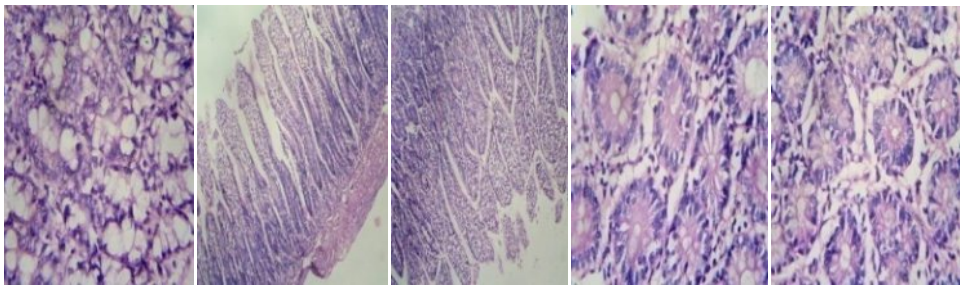
Figure 51:GROUP-IV (EASG Extract treated group)



Duodenum jejunum ileum caecum colon

- Section from duodenum shows normal epithelium. Lamina propria shows mild lymphocytic infiltrates. Muscular layer and serosa shows no significant pathology. There is no evidence of malignancy/ granuloma seen in the section studied.
- Section from jejunum shows normal epithelium. Lamina propria shows mild lymphocytic infiltrates. Muscular layer and serosa shows normal. There is no evidence of malignancy/granuloma/IBD seen in the section studied.
- Section from Ileum shows focal areas of mucosal ulceration. Lamina propria shows inflammatory infiltrates. Submucosa shows peyer's patches. Muscular layer and serosa shows mild inflammatory infiltrates.
- Section from caecum shows normal mucosa. Lamina propria shows mild lymphocytic infiltrates. Muscular layer shows no significant pathology. Serosa shows no significant pathology. There is no evidence of malignancy/granuloma / IBD seen in the section studied.
- Section from colon shows normal epithelium. Lamina propria shows mild lymphocytic infiltrates. Muscular layer and serosa shows no significant pathology. There is no evidence of malignancy/granuloma/IBD seen in the section studied.

Figure 52: GROUP-V (CHSG Extract treated group)

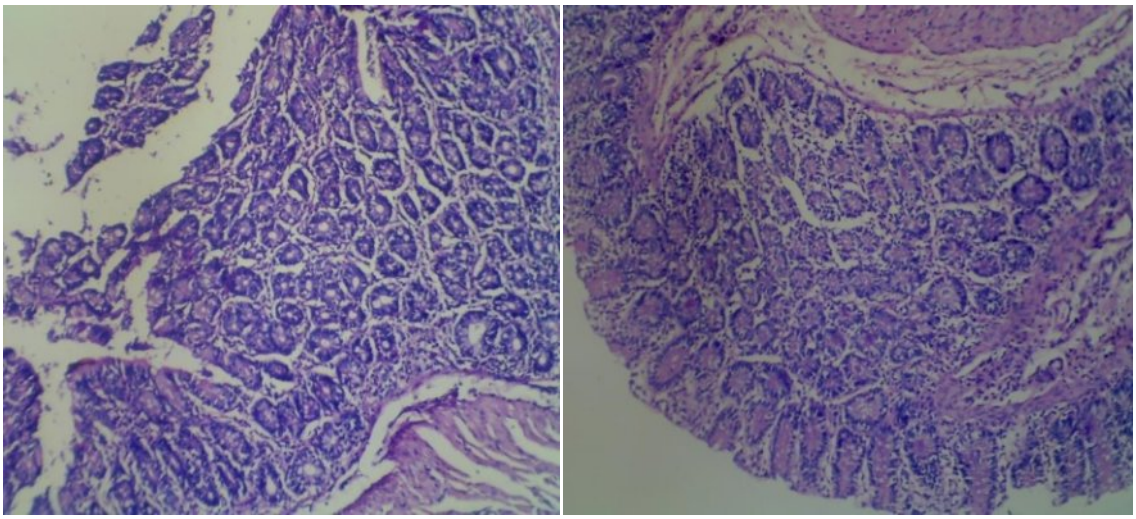


Duodenum jejunum ileum caecum colon

- Section from duodenum shows normal epithelium. Lamina propria shows moderate lymphoplasmacytic infiltrates. Submucosa shows inflammatory infiltrates. Muscular layer shows significant pathology. Serosa shows mild inflammatory infiltrates.
- Section from jejunum shows normal epithelium. Lamina propria shows mild lymphocytic infiltrates. Muscular layer and serosa shows normal. There is no evidence of malignancy/granuloma/IBD seen in the section studied.
- Section from Ileum shows normal mucosal surface. Lamina propria shows mild lymphocytic infiltrates. Submucosa shows peyer's patches. Muscular layer and serosa shows normal morphology.
- Section from caecum shows normal mucosa. Lamina propria shows edema and chronic inflammatory infiltrates. Muscular layer shows no significant pathology. Serosa shows mild inflammatory infiltrates.
- Section from colon shows normal epithelium. Lamina propria shows mild lymphocytic infiltrates. Muscular layer and serosa shows no significant pathology. There is no evidence of malignancy/granuloma/IBD seen in the section studied.

4.10.2.Acetic acid-induced enterocolitis in rats

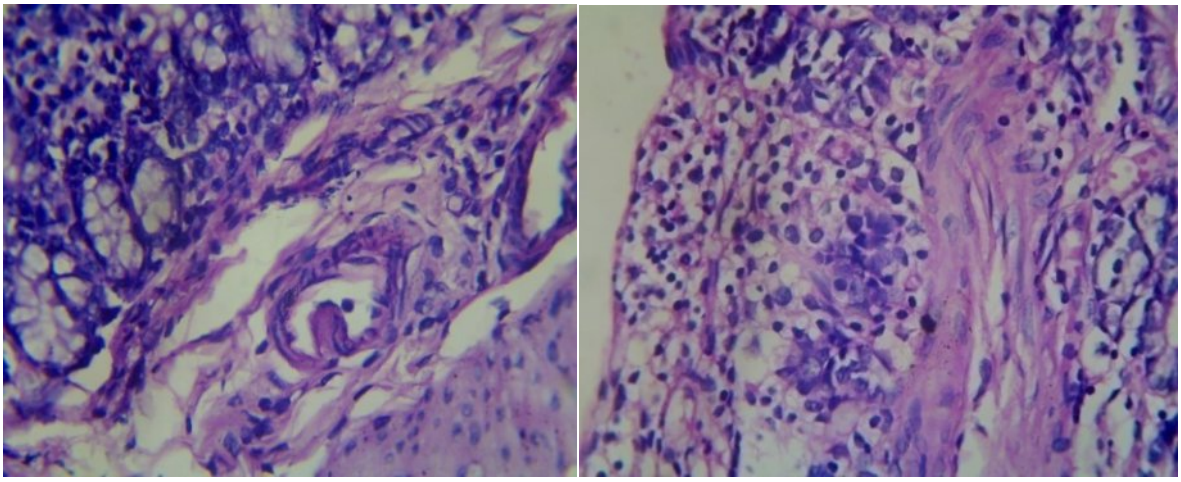
Figure 53:**GROUP-I (normal group)**



Normal Colon of rat **Normal Caecum of rat**

- Section from colon shows normal epithelium. Lamina propria shows few scattered lymphocytic infiltrates and connective tissues. There is no malignancy/granuloma seen
- Section from caecum shows normal mucosa.Lamina propria shows normal morphology.Muscular layer shows normal.There is no evidence of cryptitis and crypt abscess.

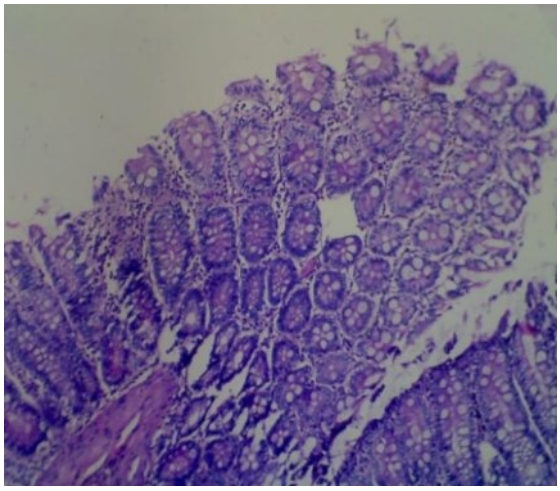
Figure 54: GROUP-II (Acetic-acid control)

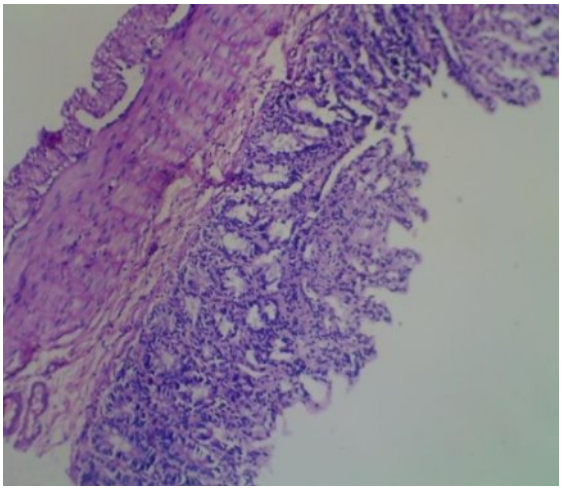


Acetic acid control of rat colon **Acetic acid control of rat caecum**

- Section from colon shows dense lymphoplasmacytic infiltrates in the lamina propria.Glands shows lymphocytes (cryptitis)
- Section from caecum shows mucosal ulceration with dense lymphoplasmacytic infiltrates in the lamina propria. Glands show lymphocytic infiltrates(cryptitis).

Figure 55: Group-III (Standard group)

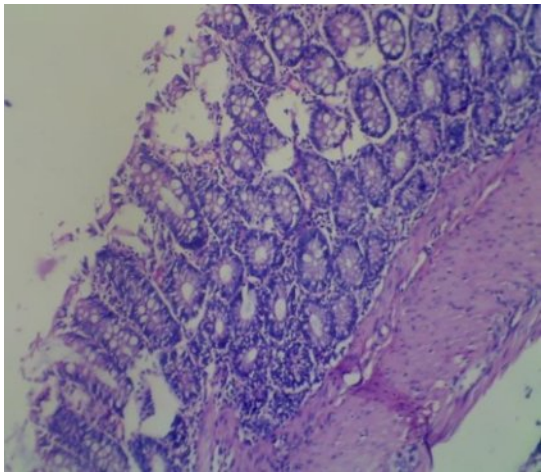


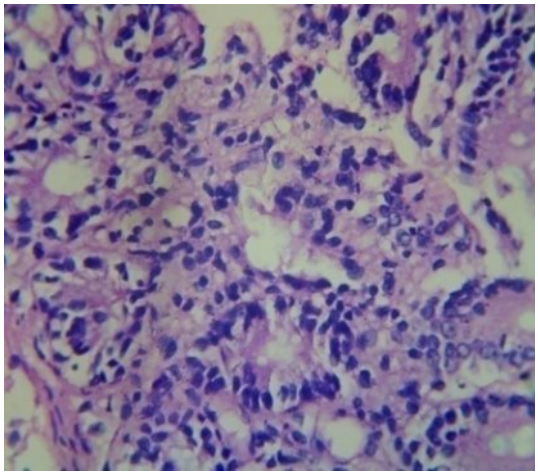


standard group of rat colon **standard group of rat caecum**

- Section from colon shows normal epithelium.Dense lymphoplasmacytic infiltrates is noted in the lamina propria.Glands show mild cryptitis.
- Section from caecum shoes normal mucosa.Lamina propria shows normal morphology. Muscular layer shows normal. There is no evidence of cryptitis and crypt abscess.

Figure 56: Group-IV (EASG extract treated group)

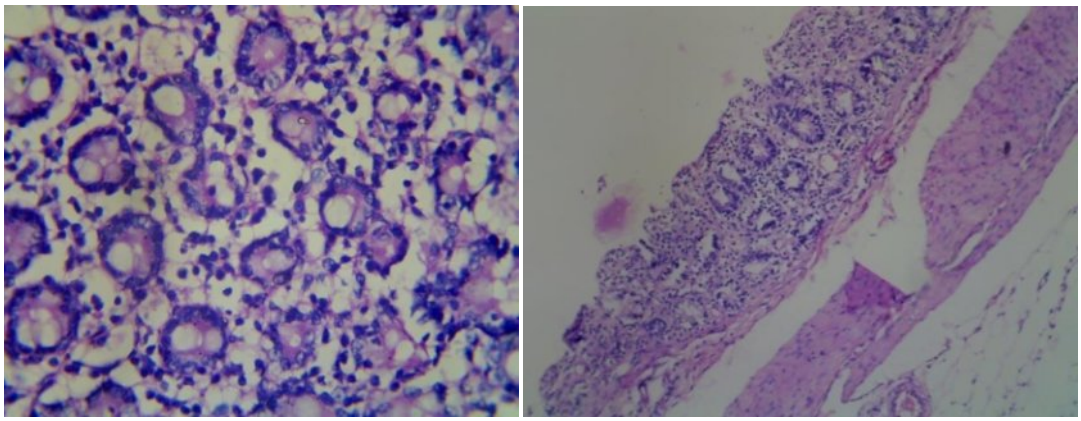




EASG extract of rat colon **EASG group of rat caecum**

- Section from colon shows normal epithelium.Dense lymphoplasmacytic infiltrates is noted in the lamina propria.Glands shows mild cryptitis.
- Section from caecum shows mild mucosal ulceration with mild scattered lymphoplasmacytic in the lamina propria.Glands show mild lymphocytic infiltration(cryptitis).

Figure57 : Group-V (CHSG treated group)



CHSG group of rat colon **CHSG group of rat caecum**

- Section from colon shows normal epithelium. Mild lymphoplasmacytic infiltrates is noted in the lamina propria. Glands show mild cryptitis.
- Section from Caecum shows mild mucosal ulceration with mild lymphoplasmacytic infiltrates in the lamina propria. Glands show mild lymphocytic infiltrations

5.DISCUSSION

The present study was aimed to assess the efficacy of chloroform and ethylacetate leaves extract of *Simarouba glauca* for its anti-inflammatory activity in indomethacin and acetic acid induced enterocolitis in wistar rats. Indomethacin, a non-selective COX inhibitor produces enterocolitis. The mechanisms of indomethacin-induced enterocolitis have not been fully illustrated, but in previous reports it was suggested that, initial epithelial damage is mediated partly by synthesis inhibition of the protective prostaglandins PGE_1, PGE_2 and prostacylin. In addition, luminal bacterial and bacterial products also contribute to the inflammatory response in the indomethacin-induced colitis model. Indomethacin induced model affect the middle portion of small intestine (jejunum and proximal ileum) and most severely the caecum,the mechanism is due to decrease level of prostoglandin and generation of free radicals which damage the mucosal integrity .

Intrarectal administration of dilute solution of acetic acid causes non-transmural inflammation characterized by increased neutrophil infiltration into the intestinal tissue, massive necrosis of mucosal and submucosal layers, vascular dilation, edema and submucosal ulceration that are noteworthy features of human colitis . Acetic acid cause damage to distal colon portion, The mechanisms by which acetic acid produces inflammation appear to involve the entry of the protonated form of the acid into epithelium, where it dissociates to liberate protons within intracellular acidification that are likely accounts for the epithelial injury.

Preliminary Phytochemical analysis

Phytochemical screening of Chloroform and Ethylacetate leaves extract of *Simarouba glauca* was carried out and the results showed the presence of carbohydrates, alkaloids, Steroids and Sterols, Glycosides, flavanoids, saponins, Tannins & phenolic compounds, proteins and amino acids.

Estimation of phytoconstituents

Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolic compound. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The contents of total phenols and flavonoids were estimated by the standard curves and expressed as Gallic acid equivalents for total phenols and quercetin equivalent for flavonoids. The amount of total

phenolic content in Ethylacetate and chloroform extract leaves of *Simarouba glauca* was found to be 118.35mg/g and 148.35mg/g respectively calculated as Gallic acid equivalent and the total Flavanoids content in the Ethylacetate and chloroform leaves extract of *Simarouba glauca* was found to be 77.05mg/g and 101.19mg/g respectively calculated as Quercetin equivalent.

High Performance Thin Layer Chromatography Study

Inorder to justify and quantify the presence of steroids, the extract was subjected to HPTLC screening against a marker compound β-sitosterol. β-sitosterol is one of the phytosterol(plant sterol) which has chemical structure similar to that of cholesterol.It provide a symptomatic relief of inflammatory bowel disease. From the results obtained from HPTLC study,it was found that CHSG & EASG contains 51.39 & 42.16 µg of β-sitosterol.

In vitro* Antioxidant Activity of ethyl acetate and chloroform leaves extract of *Simarouba glauca

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. They are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ageing, IBD, dementia etc. Therefore in the present study ,the potential of the CHSG & EASG to serve as antioxidants was assayed.

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. The free radical scavenging activity of the extract was estimated by comparing the %inhibition of CHSG &EASG with standard ascorbic acid.IC_{50} was also calculated to determine the amount of extract needed to quench 50% of radicals. A lower value of IC_{50} indicates a higher antioxidant activity. From the results obtained, IC_{50} value of ascorbic acid and CHSG & EASG was found to be 2.989µg/ml and 11.94µg/ml & 15.97µg/ml respectively. The study suggests that the extract contains compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity and it could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

The ability of CHSG &EASG to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS. ABTS assays presented as an excellent tool in determining the antioxidant activity of hydrogen donating antioxidants (Scavengers of lipid peroxyl radicals). From the

results obtained in ABTS assay it was found that the extracts scavenged ABTS radicals generated by the reaction between ABTS and potassium persulphate. The activity was found to be increased in a dose dependent manner. IC_{50} value of ascorbic acid was found to be 0.1142 µg/ml whereas 1.134µg/ml &1.160 µg/ml for CHSG and EASG extracts. Therefore the ABTS radical scavenging activity of extract at higher concentrations may have the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain-breaking reaction.

Thus from the result obtained it could be concluded that CHSG&EASG shows a good antioxidant activity which might be attributed to the presence of phytochemicals such as polyphenols. Oxygen derived free radical generation has been implicated in the etiology and pathogenesis of inflammatory bowel disease. Superoxide anion can react with nitric oxide to form peroxy nitrite anions and dismutase into hydrogen peroxidase, a reaction that is accelerated by SOD.H_2O_2 can exert its toxic effects mainly through the ferrous ion dependent formation of the highly reactive hydroxyl radicals which leads to alteration of lipids, proteins and DNA.

In order to understand the effect of EASG & CHSG extract of *simarouba glauca* on human Inflammatory bowel disease Intestinal epithelial cells, *invitro* study were conducted using cultured MTT Assay on caco2 cell lines. MTT assay is a well-established *in vitro* method for assessing cell viability and cytotoxicity against inflamed cell lines. The CTC 50 values for EASG & CHSG of *Simarouba glauca* was foun the cell lined to be 883.33±5.8 and >1000 respectively .A significant reduction in cell damage was observed when caco2 cells were pretreated with EASG and CHSG extracts when compared to control.

We have studied two experimental animal models for IBD.Intrarectal instillation of acetic acid in rats affected only the caecum and distal colon portion. The inflammation was not transmural.
Massive necrosis of mucosal and submucosal layers was observed. The mechanisms by which acetic acid produces inflammation appear to involve the entry of the protonated form
of the acid into epithelium, where it dissociates to liberate protons within intracellular acidification that most likely accounts for the epithelial injury observed. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipooxygenase pathways.
Indomethacin given subcutaneously in rats affected the middle portion of small intestine (jejunum and proximal ileum) and most severely the caecum. The inflammation was not

continuous. It showed some patches of normal tissue (skip areas). Also the necrotic foci were transmural. These findings suggest that this experimental model resemble Crohn's disease).The pathogenesis of the lesions produced by indomethacin is not clear. Pathogenic mechanism was thought to be prostaglandin-related in indomethacin enteropathy in rats. Local changes in intestinal microflora were also thought to be important and this is confirmed by reports that germ free animals do not develop lesions readily.Thus, the postulated mechanism is that altered mucosal prostaglandin synthesis compromises intestinal integrity, resulting in mucosal response to the bacterial products.

There was a significant decrease in the body weight of both the indomethacin and acetic acid induced group compared with normal group. The EASG & CHSG extracts treated group and the standard prednisolone group showed slight decrease in the body weight compared to indomethacin and acetic acid induced group (control).

. The treatment with EASG and CHSG leaves extract of *Simarouba glauca* has shown a decrease in the macroscopic scores for the inflammation. A significant decrease in MPO activity was also observed. All these observations support the findings that the EASG and CHSG leaves extract of *Simarouba glauca* was able to offer significant protection in both the models studied.

Acetic acid-induced colitis and indomethacin-induced enterocolitis simulate two different disease conditions, which are ulcerative colitis and Crohn's disease respectively. On this basis we can say that the EASG and CHSG extracts under study may be useful in treating UC as well as CD in humans.

The mechanism of development of disease in indomethacin-induced enterocolitis involves role of protective prostaglandins and intestinal pathogens. The extracts might be active due to its anti-inflammatory, cytoprotective and antimicrobial properties. Acetic acid-induced colitis involves inflammatory response initiated by the injury caused by acid. It involves stimulation of cyclooxygenase and lipooxygenase pathways and generation of inflammatory mediators like prostaglandins and leukotrienes. The extracts may have an effect on synthesis or release of these inflammatory mediators.

The prednisolone treatment has shown significant protection in both the animal models under our study. The EASG and CHSG leaves extract of *Simarouba glauca* was found comparable with prednisolone.The EASG and CHSG leaves extract of *Simarouba glauca* at doses 400 and 400 mg/kg was as good as prednisolone at the doses 10mg/kg for rats. It is possible that the extracts

acts by the same mechanism as the prednisolone i.e., by decreasing the number of neutrophils and reduction in the synthesis of inflammatory mediators.

The myeloperoxidase assay showed significant increase in MPO activity of indomethacin and acetic acid induced control group compared to normal untreated group. The Extracts treated and prednisolone treated group showed significant reduction in MPO activity compared to the control group, and from the two extracts CHSG was to be more effective in reducing MPO levels when compared to EASG.

Oxidative stress is the main cause in the pathogenesis of inflammatory bowel disease.The activity of SOD is a sensitive index in oxidative damage as it scavenges the superoxide anion to form hydrogen peroxide leading to diminish the toxic effects.The catalase is an enzymatic antioxidant that decomposes hydrogen peroxide and protects the tissue from highly reactive hydrogen peroxide through simultaneous oxidation of reduced glutathione(GSH) to oxidized glutathione(GSSH).GSH reacts with free radicals and protects from singlet oxygen,hydroxyl radicals and superoxide radical damage.Therefore reduction in the activity of these enzymes may result in a number of deleterious effects due to accumulation of superoxide radicals and hydrogen peroxide,linked with inflammatory bowel disease.Therefore evaluation of the protective effect of CHSG&EASG in rats intestinal oxidative stress parameters and antioxidant mechanisms was another objective study.In the present study,data revealed that the efficacy of ethylacetate and chloroform Extract of *Simarouba glauca* on the level of SOD, CAT, GSH, GPx and LPO in experimental animals. In IBD the activity of the SOD, CAT, GSH, and GPx were found to be increased in the ethylacetate and chloroform extract of *Simarouba glauca* in treated rats and decrease only in Idomethacin and acetic acid group treated animals. All the SOD, CAT, GSH, GPx were significantly altered with the treatment of EASG & CHSG extract of *Simarouba glauca* and prednisolone standard groups when compared with group only indomethacin and acetic acid induced animals. In this study the lipid peroxidation and was significantly increased in control group animal when compared with the group treated with EASG and CHSG as well as prednisolone standard treated group. From the *invivo* antioxidants studies it was found that CHSG extract has more *invivo* antioxidants activity when compared to EASG extract.

Histological examination of indomethacin control group showed advanced lesions as necrosis of even payers patches and fragmentation of nuclei. The EASG and CHSG extracts

treated group showed reduced intensity of lesions without any evidence of necrosis, regeneration or inflammatory reaction. Prednisolone treatment showed suppressed inflammatory reaction.

Histological examination of Acetic acid control group showed massive necrosis of the mucosa and submucosa. EASG and CHSG extracts treated group showed mild lesions, regeneration and inflammatory reaction. The prednisolone treated group showed suppressed inflammatory reaction.Among the two extracts it is evident that the CHSG extract shows more anti inflammatory action when compared to EASG extract.

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6. **CONCLUSION**

Inflammatory bowel disease (IBD) is a chronic inflammatory, idiopathic disorder of intestine with unknown etiology. Inflammatory bowel disease is of two different types; ulcerative colitis and Crohn's disease. Ulcerative colitis mainly involves inflammation of colon where as Crohn's disease is an inflammation of gastrointestinal tract from the mouth to the anus. Only 10 to 20% of people suffering from IBD have major problem that this disease itself leads to colon cancer or bowel cancer. Generation of the free radicals during the progression of the IBD has been suggested as an important factor for initiation and progression of cancer. Genetic modulation, infective agents, immunological disturbance, smoking, microorganism were found to be responsible for mucosal inflammation, hemorrhage and development of ulcers in colon.

Based on the results obtained,it can be concluded that the Ethylacetate and Chloroform extract of *Simarouba glauca* has a protective activity against experimentally induced IBD, due to its anti-inflammatory and antioxidant properties. The protective action of this plant may be due to the presence of active constituents β-sitosterol , flavanoids and polyphenols.All the Parameters of extract treated group animals have shown better results which are comparable with the standard prednisolone treated group. Possible mechanism of action may be due to decreasing number of neutrophils and reduction in the synthesis of inflammatory mediators like myeloperoxidase enzyme.

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